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— Original Article —

Somatostatin receptors and the effect of somatostatin on histamine-stimulated adenylate cyclase activity in isolated gastric glands of guinea pigs

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Summary: To investigate the mechanism of the suppressive action of somatostatin on gastric acid secretion, we determined the somatostatin binding and adenylate cyclase activity on crude membrane fractions of isolated gastric glnads of gunea pigs. The binding studies using ^{125}I-Tyr^{11} somatostatin showed the presence of somatostatin receptors with a single high affinity on crude membrane fractions. The dissociation constant (K_D) for somatostatin was 1.05±0.13 nM, and the number of binding sites (B_{max}) was 6.98±1.27 fmol/mg protein (mean±SE, n=6). The adenylate cyclase activity was increased by histamine, which was completely inhibited by 10^{-3}M cimetidine. Somatostatin non-competitively suppressed the histamine-stimulated adenylate cyclase activity in the presence of guanosine 5′-triphosphate (GTP). These results suggest that somatostatin inhibits histamine-stimulated acid secretion throught the inhibition of the adenylate cyclase system, via somatostatin receptor and guanine nucleotide binding protein, which is activated by GTP binding. *Gastroenterol Jpn 1989;24:611–618*

Key words: *adenylate cyclase activity; Gi-protein; somatostatin; somatostatin receptor*

Introduction

Somatostatin, first described as a growth hormone release inhibiting factor from the hypothalamus[1] has been shown by immunohistochemistry and radioimmunoassa to be widely distributed in the gastrointestial tract and pancreas[2]. In the stomach, many somatostatin cell processes ended on gastrin cells in the antrophyloric mucosa and on parietal cells in the oxyntic mucosa[3]. It has also been reported that somatostatin inhibited gastric acid secretion induced by histamine, gastrin, acetylcholine and food[4-6]. the suppression by somatostatin of the gastric acid secretion was alos noted in the isolated gastric glands and parietal cells[7-10].

To investigate the mechanism of the suppressive action of somatostatin on gastric acid secretion, we determined the somatostatin receptors using ^{125}I-Tyr^{11} somatostatin as a ligand and adenylate cyclase activity in the crude membrane preparation obtained from isolated gastric glands of guinea pigs, and the effects of somatostatin and guanine nucleotides were examined.

Materials and Methods

Chemicals

Adenosine 5′-triphosphate (ATP), guanosine 5′-triphosphate (GTP), 5′-guanoylyldiphosphoimide (Gpp(NH)p), creatine kinase, creatine phosphate, dithiothreitol, bacitracin, thimerosal, 2-hydroxyethyl-piperazine-N′-ethanesulfonic acid (HEPES), bovine serum albumin (Fraction V) were purchased from Sigma

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Chemical Co (St Louis, MO). Carrier-free $Na^{125}I$ (IMS-30) was purchased from the Radiochemical Centre (Amersham, England). Cimetidine was a gift from Smith Kline & Fujisawa, Co (Osaka Japan). Synthetic cyclic somatostatin 14 and Tyr^{11}-somatostatin 14 were purchased from the Peptide Institute Inc. (Osaka Japan).

Preparation of isolated gastric glands

As previously reported[9], male hartley albino guinea pigs weighing between 300-500g were deprived of food for 24 hours and then decapitated. The fundic region of the stomach was removed and rinsed several times with ice-cold 0.9% NaCl solution. The fundic mucosa separated from the muscularis was minced into small pieces using a razor blade. Minced tissue was rinsed three times in oxygenated Hanks' MEM solution (with 0.2% bovine serum albumin, 25 mM HEPES, pH 7.4), and digested with 0.05% collagenase (type IV) enzyme solution at 37°C. During digestion, the suspension was gently shaken, and perfused continuously with 100% 0_2. After 20 minutes of digstion, the suspension of isolated gastric glands was filtered through nylon mesh, and rinsed three times with ice-cold Hank's MEM solution.

The parietal cells were distinguished by size and shape of acridine orange stain by fluorescence microscopy. The average number of parietal cell in isolated gastric gland was 59.1±2.5% (mean±SE, n=15). The viability of isolated gastric glands was tested by the exclusion of 0.5% trypane blue, and more than 95% of isolated gastric glands were viable.

Somatostatin binding assay

Isolated gastric glands were homogenized in ice-cold 0.25M sucrose/3mM Tris-HC1, pH7.4, with a motor-driven Potter-Elvejhem Teflon-glass homogenizer (7 strokes at 2,000 rpm). Subcellular fraction was achieved by submitting the homogenates to differential centrifugation. The crude membrane preparation was obtained as the pellet obtained from centrifugation at 30,000 xg for 20 minutes. The resulting pellet was resuspended in 50 mM Tris-HC1 buffer and stored in liquid nitrogen and used in the following experiments. Preparation of ^{125}I-Tyr-^{11}-somatostatin was radioiodinated by a modification of the procedure of Vinik[11]. Twenty μl of 0.5 M phosphate buffer, pH7.6, were added to a small conical flask. Five μl of the solution of Tyr^{11}-somatostatin (2.5 μg in 1N acetic acid) of $Na^{125}I$. The reaction was started by the addition of 10μl of chloramin T solution (0.25 mg/ml in 50 mM phosphate buffer, pH7.6) prepared immediately prior to use. The mixture was agitated on a Vortex for 90 seconds and then 200 μl of bovine serum albumin (10 mg/1ml in 50 mM phosphate buffer pH7.6) were added. The mixture was again mixed vigorously and rapidly loaded on a column (10 × 50 mm) of carboxymethyl cellulose (CM-52, Whatman Inc., Clifton, NJ) which was previously equilibrated with a solution of 2mM ammonium acetate buffer (pH4.65). The column was then eluted with the same solution, and thirteen fractions (1.5 ml) were collected. The column was then eluted with 0.5 M ammonium acetate buffer (pH4.65) and 1 ml fractions were collected and 5 μl of each fraction were counted in a gamma counter to monitor elution of radioactivity. The hottest peak was added 40 μl of 35% hydrochloric acid and then freezed immediately. The specific activity of 176.2±2.3 Ci/g (mean±SE n=9) was calculated.

Specimens of 100 microliters of the crude membranes were incubated with ^{125}I-Try^{11} somatostatin (40000-60000 cpm) for 60 minutes at 20°C in 50 mM Tris-HC1 buffer, pH 7.4, containing 5 mM $MgC1_2$, 2 mM glycoletherdiamine tetraacetic acid (EGTA), 200 KIU/ml trasylol, 0.02 mg/ml bacitracin, 0.05 mM thimerosal and 1% bovine serum albumin in a total volume of 0.2 ml. The incubation was terminated by addition of 4 ml ice-cold 50 mM Tris-HC1 buffer, followed by rapid filtration of the mixture through Whatman GF/C glass fiber filters (Whatman. Inc) presoaked in 1% bovine serum albumin. After washing the filter twice with 5 ml of the same buffer, the radioactivity was counted using a gamma-counter. Specific binding was calculated by subtracting the nonspe-

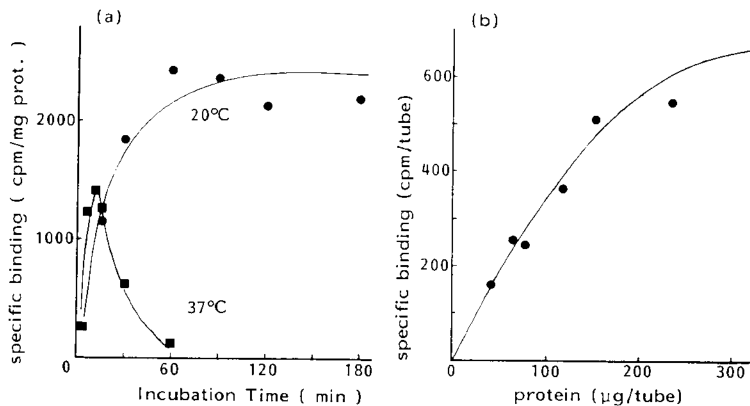

Fig. 1
(a) Effects of time and temperature on binding of ^{125}I-Tyr^{11} somatostatin to crude membrane fractions of isolated gastric glands. Crude membrane fractions (0.185mg) were incubated with 1.5nM ^{125}I-Tyr^{11} somatostatin at the designated temperature (37°C ■–■, 20°C ●–●) for the incubated time. Data are corrected for nonspecific binding.
(b) Effect of protein concentration on ^{125}I-Tyr^{11} somatostatin binding. Incubation are performed in the presence of 0.72nM ^{125}I-Tyr^{11} somatostatin for 60 minutes at 20°C. Data are corrected for nonspecific binding.

cific binding in the presence of unlabelled 10^{-6}M somatostatin.

Assay of the adenylate cyclase activity

Isolated gastric glands were sonicated in ice-cold 0.25 M socrosek/3mM Tris-HC1, pH7.4, with a Branson Sonifier, and then crude membrane was prepared by the same method. The final pellet was resuspended in 10 mM Tris-HC1 buffer, pH7.6, containing 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol, and then stored in liquid nitrogen.

Adenylate cyclase activity was estimated by determining the rate of 3′,5′-cyclic adenosine monophosphate (c-AMP) formation from ATP at 30°C in a defined system during 10 minutes of incubation. The incubation medium contained 1 mM ATP as the substrate, 1 mM EDTA, 5 mM $MgC1_2$, 10 mM theophylline as a phosphodiesterase inhibitor and the ATP regenerating system (10 mM creatine phosphate and 10 unit/ml creatine kinase) and 50 mM Tris-HC1 (pH7.8, 30°C) in a total volume of 0.25 ml. The crude membrane was appropriately diluted with 50 mM Tris-HC1 buffer (pH7.8, 30°C) containing 1 mM dithiothreitol and 1 mM EDTA. The reaction was started by addition of the crude membrane preparation, and the reaction mixture was incubated for 10 minutes at 30°C. The reaction was stopped by heating in a boiling water bath for 2 minutes. The precipitatd protein was removed by centrifugation (3,000 rpm), and samples of supernatant were assayed for c-AMP by radio-immunoassay (Yamasa cyclic-AMP assay kit). In the experiment using somatostatin, 0.1% bovine serum albumin and 0.02 mg/ml of bacitracin were added to the reaction system. The protein concentration was measured by Lowry's method[12]. Statistical analyses were made by Student's t-test and Welch's test.

Results

Somatostatin binding study

Specific binding of ^{125}I-Tyr^{11}-somatostatin was time- and temperature-dependent. At 37°C, the specific binding reached a peak after 10 minutes from the start of incubation and then rapidly declined. At 20°C, maximal binding was obtained after 60 minutes and the magnitude was about twice as large as that at 37°C (**Fig. 1a**). When increasing amounts of crude membranes were added to the incubation medium, specific binding of ^{125}I-Tyr^{11}-somatostatin increased linearly with protein concentration up to 0.2 mg protein. It then tended to reach a plateau at higher concentration (**Fig. 1b**). Further binding experiments were thus carried out at protein quantities of 0.15-0.2 mg/tube at 20°C. Maximum binding of ^{125}I-Tyr^{11}-somatostatin amounted to 0.1% of the total radio-activity available. The specific binding increased and became saturated as the concentration of ^{125}I-Tyr^{11}-somatostatin. The scatchard plot of this saturation curve was linear, indica-

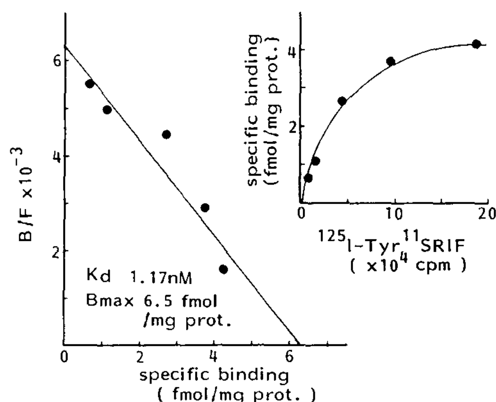

Fig. 2 Scatchard analysis of ^{125}I-Tyr^{11} somatostatin binding to crude membrane fraction of isolated gastric glands. Each plot represents the means of triplicate determinations from a typical experiment. The slope of the plot gives a K_D of 1.17nM, and the intercept on the abscissa gives a binding capacity (B_{max}) of 6.50 fmol/mg protein. The mean of six different experiments yielded a K_D value of 1.05±0.13nM and a B_{max} of 6.98±1.27 fmol/mg protein.

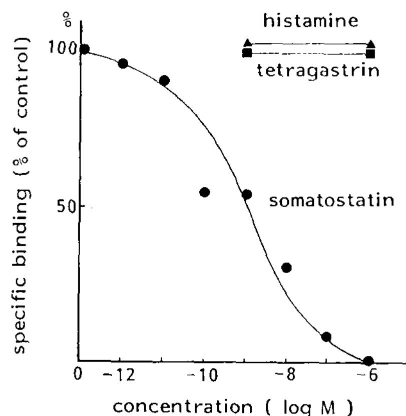

Fig. 3 Inhibition of ^{125}I-Tyr^{11} somatostatin binding to crude membrane fractions of isolated gastric glands in the presence of increasing concentrations of somatostatin. ^{125}I-Tyr^{11} somatostatin was incubated at 20°C for 60 minutes in the presence of increasing concentrations of somatostatin, at a concentration of 10^{-6}M. Resuts were expressed as the percentage of specific binding in the absence of somatostatin. Each point represents the mean of three different experiments triplicate.

tive of a single population of saturable binding sites with a dissociation constant (K_D) of 1.05±0.13 nM and a number of binding sites (B_{max}) of 6.98±1.27 fmol/mg protein (mean±SE, six independent experiments) (**Fig. 2**). The rate of dissociation was determined from experiments in which crude membranes were preincubated with ^{125}I-Tyr^{11}-somatostatin for 60 minutes at 20°C, followed by addition of unlabeled 10^{-6}M somatostatin. The specific activity rapidly declined with a half-time of 12 minutes. The specific binding was dose-dependently suppressed by unlabeled somatostatin with IC_{50} of 10^{-9}-10^{-8} M. Tetragastrin and histamine had no effect on the specific binding. These observations suggest that the receptor specific to somatostatin is present in the crude membrane fraction (**Fig. 3**).

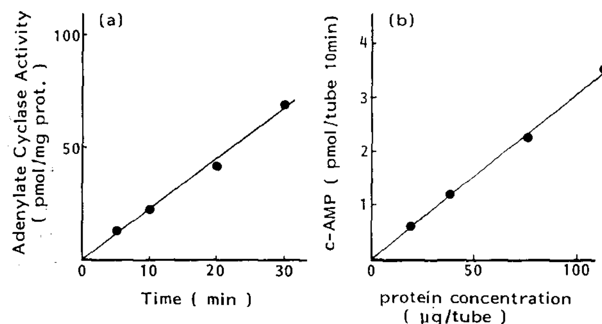

Fig. 4 (a) Time course of adenylate cyclase activity. The formation of c-AMP of the enzyme specimen from isolated gastric glands was assayed at 30°C for time points up to 30 minutes. Values are means, n=4. (b) Effect of protein concentration on adenylate cyclase activity. The formation of c-AMP from isolated gastric glands was assayed at protein concentrations from 0.019mg to 0.102mg. Values are means, n=4.

Adenylate cyclase activity

The c-AMP formation of crude membranes increased linearly up to 30 minutes after start of the incubation (**Fig. 4a**). When increasing amounts of crude membranes were added to the incubation medium, c-AMP formation increased linearly with a protein concentration of up to 0.1 mg/tube (**Fig. 4b**). Therefore, the protein concentration of assay tubes was fixed at 0.03-0.05 mg/tube in the following experiments. The mean basal activity of adenylate cyclase was calculated to be 13.18±0.70 pmol/mg protein/10 minutes (mean±SE, n=8), and the Michaelis-Menten constant (Km) was

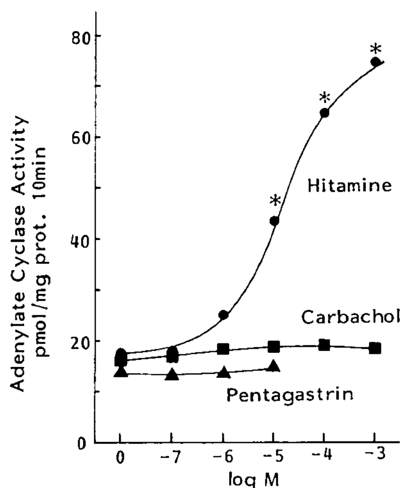

Fig. 5 Effects of gastric acid secretagogues on adenylate cyclase activity. Adenylate cyclase activity was stimulated by histamine (●-●), carbachol (■-■) and pentagastrin (▲-▲). Values are means, n=4. (*;P<0.001)

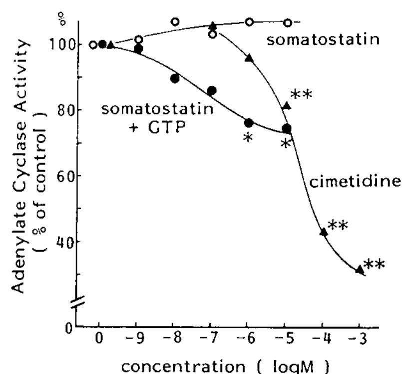

Fig. 6 Suppression of the histamine-stimulated adenylate cyclase activity by cimetidine and somatostatin. Cimetidine (▲-▲), somatostatin (with GTP 10^{-5}M ●-●, without GTP ○-○). Results were expressed as the percentage of 10^{-4}M histamine-stimulated adenylate cyclase activity. Values are means, n=4 (*;P<0.05, **;P<0.01)

Table 1 Effects of guanine nucleotide and sodium fluoride (NaF) on adenylate cyclase activity

		Adenylate Cyclase Activity pmol/mg protein/10 minutes	
control		13.18± 0.70	(n=8)
+ GTP	10^{-5}M	33.98± 1.19	(n=4)*
+ Gpp(NH)p	10^{-5}M	32.91± 1.03	(n=4)*
Histamine	10^{-4}M	52.38± 1.01	(n=4)*
+ GTP	10^{-5}M	80.42± 2.78	(n=4)*
+ Gpp(NH)p	10^{-5}M	118.86± 4.84	(n=4)*
NaF	10^{-2}M	166.74±17.84	(n=4)*

All results were expressed as mean±SE.
*; P<0.001.

7.54±2.10 × 10^{-6}M, and V max was 16.44±1.54 pmol/mg protein/10 minutes (mean±SE, n=4) by Lineweaver-Burk analysis.

Histamine increased the adenylate cyclase activity in a dose-dependednt manner at the range of 10^{-6}M to 10^{-3}M. At 10^{-3}M histamine, the adenylate cyclase activity was maximally increased to 74.8±2.2 pmol/mg protein/10 minutes (mean±SE, n=4). Carbachol (10^{-3}M), and pentagastrin (10^{-5}M), however, did not significantly alter the adenylate cyclase activity (**Fig. 5**). Both GTP and its derivative Gpp(NH)p significantly enhanced the basal or 10^{-4}M histamine-stimulated adenylate cyclase activity, and Gpp (NH) p was most effective (P<0.01). Sodium fluoride (10^{-2}M) also significantly increased adenylate cyclase activity to 166.7±17.84 pmol/mg protein/10 minutes (mean±SE, n=4). (**Table 1**).

Cimetidine, H_2 receptor antagonist, dose-dependently suppressed the adenylate cyclase activity stimulated by 10^{-4}M histamine, and 10^{-3}M cimetidine completely inhibited to nearly the basal level. The IC_{50} of cimetidine was in the micromolar range. In the case of somatostatin, when GTP was not present in the reaction system, neither the non-stimulated nor the histamine-stimulated adenylate cyclase activity was suppressed. Even in the presence of 10^{-5}M GTP, the non-stimulated adenylate cyclase activity was not sppressed by somatostatin. However, the histamine-stimulated adenylate cyclase activity in the presence of 10^{-5}M GTP was significantly and dose-dependently depressed by somatostatin. The maximal inhibition was attained at a somatostatin concentration of 10^{-5}M. The suppressive effect of somatostatin, however, was 32.0±3.1% (mean±SE, n=6), weaker than that of cimetidine (**Fig. 6**). This suppressive action of somatostatin was enhanced by increasing the concentrations of GTP (**Fig. 7**). To investigate the

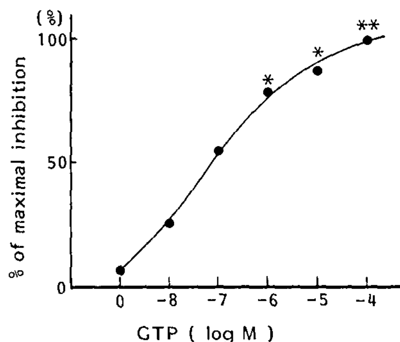

Fig. 7 Effect of GTP concentration on somatostatin inhibition of histamine-stimulated adenylate cyclase activity. Adenylate cyclase activity was stimulated by histamine 10^{-4} at the concentration of GTP ranging from none to 10^{-4}M. Inhibition by somatostatin 10^{-6}M was assayed at each concentration of GTP. Results were expressed as the percentage of maximal inhibition in the presence of GTP 10^{-4}M. Values are means, n=4. (*;P<0.01, **;P<0.001)

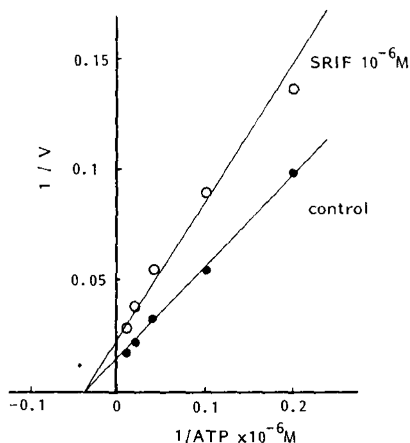

Fig. 8 Lineweaver-Burk analysis of somatostatin inhibition on histamine-stimulated adenylate cyclase activity. Each plot is the mean of triplicate determinations from a typical experiment. In the presence of GTP 10^{-5}M, histamine 10^{-4}M stimulated-adenylate cyclase activity were assayed using ATP concentrations from 0.005mM to 0.1mM ; control (●–●), somatostatin (○–○).

suppressive action of somatostatin, Lineweaver-Burk plots in the presence and absence of 10^{-6}M somatostatin were analyzed. The result showed two linear lines crossing on the abscissa, thereby suggesting the noncompetitive nature of the inhibitory action of somatostatin (**Fig. 8**).

Discussion

As possible mechanisms of the suppressive action of somatostatin on gastric acid secretion, the direct action on parietal cells and the indirect action via the suppression of gastrin secretion from antral gastrin cells have been proposed. Somatostatin inhibited the gastric acid secretion without changing the serum gastrin level in dogs with gastric fistulae and Heidenhain pouches[6]. Also somatostatin suppressed the intracellular accumulation of ^{14}C-aminopyrine induced by histamine in isolated gastric glands[7,8]. Specific somatostatin receptors have been investigated mostly in the central nervous system and pancreas, and the gastric mucosa has been used only in a few studies. In this study somatostatin possessed specific binding sites and inhibited adenylate cyclase activity on the crude membrane fractions of gastric glands. These results suggest that the major action of somatostatin may be present in the histamine receptor - adenylate cyclase system via somatostatin receptor of the parietal cells.

It has been previously shown that somatostatin binding sites are present on rat stomach[13], isolated parietal cells of rat[14,15], rabbit gastric mucosa[16] and human gastric mucosa[17]. Reyl reported that somatostatin binding sites occurred mostly in cytosol fraction in the isolated gastric cells from rat, because the maximal binding sites (B_{max}) for the cytosol fraction was found to be about 10 times greater than for the membrane fraction[15]. However, most other studies using the central nervous sytem suggested that the binding sites of somatostatin presented in the membrane fractions[18-20], which agrees with the results of our study.

The value of K_D obtained in this study was similar to those reported for membrane fractions, but the value of B_{max} was considerably smaller than that reported for the human gastric mucosa[17]. As observed in this study, the somatostatin receptors have been reported to consist of a single population of high affinity receptors, in most studies using the membrane

fraction from gastric mucosa[17], brain[18], pituitary gland[19,20], adrenal glomerulosa zone[21], and pancreatic acinar cell[22]. Although the gastric glands contained mixed cell populations, we suggest that somatostatin receptor with a single population exists in the gastric glands.

It has been established that adenylate cyclase system is a complex consisting of three elements, receptor, stimulatory G-protein (Gs-protein) and the catalytic unit, and also that the catalytic unit is activated via Gs-protein by the binding of hormone and the receptor[23]. Gs-protein is also known as guanine nucleotide binding protein and activates the catalytic unit by converting guanosine 5′-diphosphate (GDP) to GTP. Thus GTP or its derivative Gpp(NH)p activates the adenylate cyclase activity. In experiments using tissue preparations obtained by sonication of the parietal cells isolated from rats, GTP was found not to alter the adenylate cyclase activity, while Gpp(NH)p potentiated the adenylate activity stimulated by histamine[24]. In the present study in which the isolated gastric glands of guinea pigs were used, both GTP and Gpp(NH)p enhanced not only non-stimulated but also histamine-stimulated adenylate cyclase activities. Be that as it may, the result of the present study indicates the important roles of GTP and its derivative in the histamine receptor - adenylate cyclase system, and provides indirect evidence for the existence of Gs-protein.

It is also thought that adenylate cyclase has inhibitory receptors related to inhibitory G-protein (Gi-protein), and that it suppressed adenylate cyclase activity via this Gi-protein[25]. Somatostatin had no effect on the forskolin stimulated adenylate cyclase activity in cyc^- lymphoma cell membrane preparation (a mutant strain having no Gs-protein) when guanine nucleotides were not present[26]. Islet-activating protein (IAP) which ADP-ribosylates the Gi-protein, blocked the suppressive action of somatostatin[27]. Thus, the existence of the inhibitory pathway consisting of the somatostatin receptor - Gi-protein - catalytic unit has been postulated.

As described in Results, the suppressive action of somatostatin on the adenylate cyclase activity in isolated gastric glands on the adenylate cyclase activity in isolated gastric glands required GTP, and was dependent upon the GTP concentration. This strongly suggests that somatostatin inhibits the adenylate cyclase activity via Gi-protein. However, histamine stimulated adenylate cyclase activity without GTP. This result may be related to the different sensitivity of guanine nucleotide binding protein to GTP[28].

Using in vivo experimental systems, the inhibitory action of somatostatin on histamine stimulated gastric acid secretion was reported to be either competitive[29] or non-competitive[5]. Thus, no conclusion has been reached by in vivo studies. However, using gastric glands and isolated gastric cells, the inhibitory effects of somatostatin was mostly reported the non-competitive nature[7,10]. In the present study, Lineweaver-Burk analysis indicated that somatostatin inhibited histamine-stimulated adenylate cyclase activity in a non-competitive manner.

In summary, our results support the conclusion that somatostatin receptor occupancy is coupled to inhibition of adenylate cyclase activity by a guanine nucleotide-binding protein.

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