INTRODUCTION

The central role of histamine in gastric acid secretion has been studied extensively (1-6). Gastric histamine is released from enterochromaffin-like (ECL) cells in rats and subsequently stimulates acid secretion via the activation of histamine H2 receptors on parietal cells (7, 8). Histamine also stimulates the secretion of acid in the stomach via the activation of adenylate cyclase and an increase in the intracellular level of 3’, 5’-cyclic adenosine monophosphate (cAMP) (1, 2, 4). Because cAMP is degraded into inactive metabolites via hydrolysis by phosphodiesterase (PDE), it is possible that PDE affects the response to histamine by altering the levels of this nucleotide. Indeed, the acid response to histamine was reportedly potentiated by theophylline (4).

At present, the PDE in mammalian tissues has been subdivided into 11 isozymes, each derived from separate gene families and having pharmacologically distinct roles (9). PDE1 to PDE5 have been well characterized, and selective inhibitors of these isozymes are used for the treatment of heart disease, depression, asthma, inflammatory disease, and erectile dysfunction (10-12). To disclose the profiles of the subtype-selective PDE inhibitors would be helpful for eliminating the adverse influences of these agents in the body, including the gastrointestinal tract. We recently found using isolated mouse stomachs that the response of HCO3- to nitric oxide (NO) was regulated by both PDE1 and PDE5, while other subtypes of PDE had no effect (13). However, it remains unexplored which PDE isozyme(s) is involved in the regulation of gastric acid secretion.

In the present study, we examined the effects of subtype-selective inhibitors of PDE1 to PDE5 on the secretion of acid in response to histamine in the isolated mouse stomach and investigated which isozymes of PDE are involved in the local regulation of gastric acid secretion. Since the release of histamine from ECL cells is known to be regulated by cAMP, in addition to Ca2+ (4, 14), we also examined the influences of these PDE inhibitors on acid secretion and histamine release induced by pituitary adenylate cyclase activating polypeptide (PACAP), a biologically active neuropeptide with a potent stimulatory effect on histamine release from enterochromaffin-like cells.

Key words: gastric acid secretion, phosphodiesterase isozyme, histamine, parietal cell, enterochromaffin-like cell, isolated mouse stomach

MATERIALS AND METHODS

Animals

Male DDY mice weighing 25-30 g (SLC, Japan) were used in all experiments. The animals, kept in stainless steel cages with...
raised mesh bottoms, were deprived of food but allowed free access to tap water for 18 h before the experiments. All experimental procedures used were carried out in accordance with the Helsinki Declaration and have been approved by the Committee for Animal Experimentation established by Kyoto Pharmaceutical University.

Measurement of gastric acid secretion

Under deep diethyl ether anesthesia, the mice fasted for 18 h were killed and the abdomen was opened by a midline incision. The whole stomach was isolated and transferred into an organ bath containing HCO₃⁻ Ringer’s solution (mmol/L: Na⁺, 140; Cl⁻, 120; K⁺, 5.4; Mg²⁺, 1.2; Ca²⁺, 1.2; HPO₄²⁻, 1.4; H₂PO₄⁻, 2.4; HCO₃⁻, 25; glucose 10; indomethacin 0.001) gassed with 95% O₂/5% CO₂ and the lumen was perfused with unbuffered saline (mmol/L: Na⁺, 154; Cl⁻, 154) gassed with 100% O₂ (Fig. 1). The solutions were warmed at 37°C, while the osmolality of both solutions was approximately 308 mOsm/kg. Acid secretion was measured at pH 5.4 using a pH-stat method (Comtite-980, Hirunana industries, Ibaraki, Japan) with 2 mmol/L NaOH. Measurements were made every 10 min starting at least 1 h after setting the stomach in the organ bath. After the rate of secretion had stabilized for 60 min, the following agents were added to the serosal solution; histamine (10⁻⁶ to 10⁻⁴ M), PACAP (10⁻¹ M), isobutylmethylxanthine (IBMX: 10⁻⁵ to 10⁻⁴ M), vinpocetine (PDE1 inhibitor; 10⁻⁴ M), EHNA (PDE2 inhibitor; 10⁻⁵ M), cilostamide (PDE3 inhibitor; 10⁻⁴ M), rolipram (PDE4 inhibitor; 10⁻⁵ M), and zaprinast (PDE5 inhibitor; 10⁻⁴ M). The doses of these inhibitors were chosen according to published papers (13, 19) to affect the respective PDE isozyme in the stomach and duodenum. In some cases, the effects of EHNA, cilostamide and rolipram on the response to histamine and PACAP were examined; the PDE inhibitors were added 30 min before the latter agents. In addition, the effect of famotidine (a histamine H₂-receptor antagonist; 10⁻⁵ M) on the responses to histamine and IBMX was also examined; famotidine was added serosally 30 min before these agents.

Determination of luminal histamine content

The amount of histamine released in the lumen was determined in the isolated mouse stomach and after treatment with DMSO or subtype-selective PDE inhibitors. After basal acid secretion had stabilized, the perfusate (unbuffered saline) was collected for 1 h after the serosal addition of DMSO, or subtype-selective PDE inhibitors (10⁻⁵ M). The amount of histamine in the gastric perfusate was measured by enzyme immunoassay (Histamine EIA kit, Immunotech, Marseille, France).

Measurement of intracellular levels of cAMP

The isolated mouse stomach was treated with serosal addition of histamine (10⁻⁴ to 10⁻² M), PACAP (10⁻⁷ M) with or without IBMX (3x10⁻⁵ M), EHNA (10⁻⁷ M), cilostamide (10⁻⁶ M) or rolipram (10⁻⁵ M) for 5 min. In the combined treatment, PDE inhibitors were added 5 min before histamine or PACAP. The tissue was homogenized in 2 ml of 5% trichloracetic acid in a tissue homogenizer on dry ice (0–4°C). The precipitate was then extracted 4–5 times with 2 volumes of ether, and the ether fractions were collected and removed from the aqueous layer by heating the sample to 70°C for 5 min. Levels of cAMP were measured using a cAMP enzyme immunoassay kit (Cayman Chemical Co., Ann Arbor, MI).

Preparation of drugs

Drugs used were histamine, famotidine (Nacalai tesque, Kyoto, Japan), isobutyl- methylxanthine (IBMX), vinpocetine, EHNA ((erythro-9-(2-Hydroxy-3-nonyl) adenine hydrochloride), cilostamide, rolipram, zaprinast (Aldrich, Milwaukee, WI), and pituitary adenylate cyclase activating polypeptide (PACAP: Peptide Institute, Osaka, Japan). Histamine and PACAP were dissolved in saline, while famotidine was suspended in 0.5% carboxymethylcellulose solution (CMC: Wako, Osaka, Japan). Other agents were dissolved in dimethyl sulfoxide (DMSO: Wako) and diluted with distilled water to desired concentrations. All agents were prepared immediately before use and added to the serosal solution.

Statistical analysis

Data are expressed as the mean±SE for 4–6 mice. Statistical analyses were performed with a one-way analysis of variance (ANOVA) followed by the Dunnett multiple comparison test or, when appropriate, Student-t tests, and values of P<0.05 were considered significant.

RESULTS

Effects of histamine and IBMX on acid secretion in isolated stomach

The isolated mouse stomach consistently secreted acid at rates of 0.3–0.7 µEq/10 min (basal secretion) in the absence or presence of saline, 0.5% CMC, or 0.1% DMSO, a solvent for the agents used (data not shown). The isolated mouse stomach responded to
the serosal addition of histamine (10^{-6}~10^{-4} M) with an increase of acid secretion in a concentration-dependent manner, and the effect at 10^{-4} M reached a level about 7 times greater than basal values; the net total acid output was 12.7±2.6 µEq/h (Figs. 2A and 2B). The acid secretion induced by histamine (10^{-4} M) was almost totally attenuated by the addition of famotidine (10^{-5} M) to the serosal solution, the inhibition being 92.1%.

Likewise, the application of IBMX (10^{-3}~10^{-4} M) increased the secretion of acid in a concentration-dependent manner, the net total acid output at 10^{-4} M being 5.2±0.8 µEq/h (Figs. 3A and 3B). The stimulatory effect of IBMX (10^{-4} M) was all but attenuated by pretreatment of the tissue with famotidine (10^{-4} M), the net total acid output being 1.6±0.2 µEq/h, which was almost equivalent to that observed in the control tissue without IBMX treatment.

We further examined whether IBMX causes a potentiation of the response to histamine. The serosal application of histamine (10^{-4} M) slightly increased acid secretion in the isolated mouse stomach, though the net total acid output was not significantly different from the control (Figs. 4A and 4B). Likewise, IBMX (3x10^{-5} M) by itself slightly increased the rate of acid secretion, the net total acid output being 3.5±0.5 µEq/h, which was significantly greater than that observed in the control tissue. When these agents were added together, the secretion of acid was markedly increased to a maximal value of 2.8±0.4 µEq/10 min; the net total acid output was 7.8±0.7 µEq/h, significantly greater than that induced by histamine or IBMX alone.

Levels of cAMP in the isolated mouse stomachs were 310±22 pmol/g tissue under basal conditions. The serosal addition of histamine at 10^{-6} did not significantly affect intracellular cAMP content but that at 10^{-4} M significantly increased it to 724±81 pmol/g tissue, approximately 2.3 times the values observed in saline-treated control stomachs (Fig. 5). However, the response to histamine at 10^{-4} M was significantly enhanced by the nonselective PDE inhibitor IBMX (3x10^{-4} M), the value being 682±59 pmol/g tissue, significantly greater than that obtained in saline-treated stomachs. IBMX alone at this dose slightly increased the intracellular cAMP content of mouse stomachs (35.2%)(data not shown).

Effects of various PDE inhibitors on gastric acid secretion and histamine release

The nonselective PDE inhibitor IBMX at a high dose (10^{-4} M) significantly increased acid secretion and potentiated the stimulatory action of histamine in the mouse stomach. To

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**Fig. 2.** Effect of histamine on acid secretion in isolated mouse stomachs. Histamine (10^{-6}~10^{-4} M) was added to the serosal solution. Famotidine (10^{-5} M) was added to the serosal solution 30 min before histamine (10^{-4} M). (A) Data are presented as the mean ±SE of values determined every 10 min from 4-6 mice. (B) Data show the total net acid output for 1 h after the addition of histamine and are presented as the mean±SE for 4-6 mice. * Significant difference from histamine alone at 10^{-4} M, at P<0.05.

**Fig. 3.** Effect of IBMX on acid secretion in isolated mouse stomachs. IBMX (10^{-5}~10^{-4} M) was added to the serosal solution. Famotidine (10^{-5} M) was added to the serosal solution 30 min before IBMX (10^{-4} M). (A) Data are presented as the mean±SE of values determined every 10 min from 4-6 mice. (B) Data show the total net acid output for 1 h after the addition of IBMX and are presented as the mean±SE for 4-6 mice. Significant difference of P<0.05: * from control; # from IBMX (10^{-4} M) alone.
Fig. 4. Effects of histamine and IBMX, either alone or in combination, on acid secretion in isolated mouse stomachs. Histamine ($10^{-6}$ M) or IBMX ($3\times10^{-5}$ M) was added, either alone or in combination, to the serosal solution. In the combined administration, IBMX ($3\times10^{-5}$ M) was added to the serosal solution 30 min before histamine. (A) Data are presented as the mean±SE of values determined every 10 min from 4-6 mice. (B) Data show the total net acid output for 1 h after the addition of histamine and are presented as the mean±SE for 4-6 mice. Significant difference at $P<0.05$; * from control; # from histamine alone at $10^{-6}$ M.

Fig. 5. Effect of histamine on mucosal cAMP content in isolated mouse stomachs, in the absence or presence of IBMX. Histamine ($10^{-6}$ and $10^{-4}$ M) was added to the serosal solution. IBMX ($3\times10^{-5}$ M) was added to the serosal solution 30 min before histamine. Data show mucosal cAMP content 5 min after the addition of histamine and are presented as the mean±SE for 5-8 mice. Significant difference at $P<0.05$; * from saline, # from vehicle.

Fig. 6. Effects of various subtype-selective PDE inhibitors on acid secretion in isolated mouse stomachs. Vinpocetine (PDE1 inhibitor), EHNA (PDE2 inhibitor), cilostamide (PDE3 inhibitor), rolipram (PDE4 inhibitor) or zaprinast (PDE5 inhibitor) was added to the serosal solution at a concentration of $10^{-5}$ M. Data show the total net acid output for 1 h after the addition of each agent and are presented as the mean±SE for 4-6 mice. Significant difference of $P<0.05$; * from control; # from rolipram alone.
determine which PDE isozyme(s) are involved in the regulation of gastric acid secretion, we examined the effects of various subtype-selective PDE inhibitors on the secretion of acid and the luminal release of histamine.

Among the subtype-selective PDE inhibitors examined, only the selective PDE4 inhibitor rolipram by itself significantly increased the basal rate of acid secretion, the net total acid output at a dose of 10⁻⁵ M being 3.1±0.2 µEq/h (Fig. 6). Neither vinpocetine (the selective PDE1 inhibitor), EHNA (the selective PDE2 inhibitor), cilostamide (the selective PDE3 inhibitor) nor zaprinast (the selective PDE5 inhibitor) at 10⁻⁵ M had a significant effect on basal acid secretion in the stomach. The acid stimulatory effect of rolipram was totally inhibited by the co-addition of famotidine at 10⁻⁵ M, similar to that of IBMX.

To further confirm the involvement of endogenous histamine in the acid response to PDE inhibitors, we examined the effects of various PDE inhibitors (10⁻⁵ M) on luminal histamine output in the isolated mouse stomach. The amount of luminal histamine in control stomachs without any treatment was 37.8±14.1 ng/h (Fig. 7). Neither vinpocetine nor zaprinast had any effect on histamine output in the stomach. Although both EHNA and cilostamide slightly increased histamine output in the lumen, the values were not significantly different from control levels. However, the PDE4 inhibitor rolipram markedly enhanced luminal histamine output, the value being 87.5±10.8 ng/h, significantly greater than that obtained in control stomachs.

**Fig. 7.** Effects of various subtype-selective PDE inhibitors on the luminal release of histamine in isolated mouse stomachs. Vinpocetine (PDE1 inhibitor), EHNA (PDE2 inhibitor), cilostamide (PDE3 inhibitor), rolipram (PDE4 inhibitor) or zaprinast (PDE5 inhibitor) was added to the serosal solution at a concentration of 10⁻⁵ M. Data show the amount of luminal histamine released in isolated mouse stomachs for 1 h after the addition of each agent and are presented as the mean±SE for 4-6 mice. * Significant difference from control (vehicle), at P<0.05.

**Fig. 8.** Effects of histamine (A) and PACAP (B) on acid secretion in isolated mouse stomachs, in the absence or presence of subtype-selective PDE inhibitors. Histamine (10⁻⁶ M) or PACAP (10⁻⁷ M) was added to the serosal solution. EHNA (PDE2 inhibitor), cilostamide (PDE3 inhibitor) or rolipram (PDE4 inhibitor) was added to the serosal solution 30 min before histamine or PACAP. Data show the total net acid output for 1 h after the addition of each agent and are presented as the mean±SE for 4-6 mice. Significant difference at P<0.05; * from control; # from vehicle; $ from rolipram alone.

Effect of various PDE inhibitors on changes in acid secretion and cAMP content induced by histamine or PACAP

The nonselective PDE inhibitor IBMX significantly enhanced the stimulatory effect of histamine on acid secretion. In addition, the PDE4 inhibitor rolipram stimulated acid secretion with a concomitant increase in luminal histamine output. PACAP is known to stimulate acid secretion by increasing the amount of histamine released from ECL cells (17, 18). In the present study, rolipram by itself increased acid secretion with a concomitant increase in histamine output, while both EHNA and cilostamide slightly but insignificantly augmented acid secretion and histamine output. Then, we further examined the effects of these inhibitors on changes in acid secretion and intracellular cAMP content induced in the stomach by histamine or PACAP.
1. Acid secretion

As shown in Fig. 8, histamine (10^4 M) added to the serosal solution caused a slight increase in acid secretion. This response was not affected by either EHNA (10^-5 M) or cilostamide (10^-5 M) but markedly potentiated by rolipram (10^-5 M); the net acid output was 6.2±0.8 μEq/h, significantly greater than that induced by histamine alone. The acid response to histamine plus rolipram was markedly attenuated by the co-addition of cAMP (10^-5 M), the inhibition being 83.9%. Likewise, the secretion of acid was minimally increased in response to PACAP at a concentration of 10^-7 M, and this response was markedly enhanced by the co-addition of rolipram, while neither EHNA nor cilostamide had any effect. The enhanced response induced by PACAP plus rolipram was also greatly inhibited by the co-addition of cAMP, the inhibition being 85.4%.

2. cAMP content

Levels of cAMP in whole isolated mouse stomach without any treatment were 400–500 pmol/g tissue. Serosal addition of histamine (10^4 M) or PACAP (10^-7 M) alone did not significantly affect the levels of cAMP in the stomach (Figs. 9A and 9B). The effects of these agents remained unchanged in the presence of the PDE2 inhibitor EHNA (10^-5 M) or the PDE3 inhibitor cilostamide (10^-5 M), but were markedly potentiated by the PDE4 inhibitor rolipram (10^-5 M). Notably, PACAP in the presence of rolipram increased cAMP content to a value 4 times greater than that induced by this agent alone (Fig. 9B).

DISCUSSION

Endogenous histamine plays an important role in the local mechanism of acid secretion under normal and damaged stomachs (1-6), and this response is regulated intracellularly mainly by cAMP (1, 2). In the present study, we confirmed in the isolated mouse stomach in vitro that the secretion of acid was stimulated by histamine and PACAP, both mediated by histamine H2-receptors. We also observed that the histamine-induced acid secretion was potentiated by not only IBMX but also rolipram, a PDE4 inhibitor, but not inhibitors of other PDE isozymes. In addition, the acid response to PACAP was increased by rolipram but not other PDE inhibitors, with a concomitant increase in histamine release as well as intracellular cAMP content. It is assumed that among the PDE isozymes, PDE4 is involved in the local regulation of acid secretion via the degradation of cAMP, probably at different cell levels, by increasing acid production in parietal cells and enhancing histamine release from ECL cells.

The acid response to histamine is potentiated by inhibition of PDE, the major enzyme that degrades cyclic nucleotides such as cAMP into inactive components (4). PDE has been subdivided into 11 isozymes (9), yet which type(s) are responsible for degrading cAMP remains unknown. In the present study, we examined the effects of subtype-selective PDE inhibitors on acid secretion in mouse stomachs and investigated which PDE isozymes are responsible in the local regulation of this secretion. First, we observed that histamine stimulated acid secretion in the isolated mouse stomach, and this response was totally inhibited by cimetidine and increased by IBMX, confirming that the secretory response is intracellularly mediated by cAMP via the activation of histamine H2-receptors (2, 4, 5). The potentiating effect of IBMX was mimicked by the PDE4 inhibitor rolipram, but not inhibitors of other PDE isozymes, and the enhanced response was accompanied by an increase in intracellular cAMP content. It was also found that rolipram by itself slightly but significantly increased acid secretion, although the acid output was approximately 1/4 of that induced by histamine at a maximal dose. Since the acid response to rolipram was totally inhibited by cimetidine and since this PDE inhibitor also increased the luminal release of histamine, it is assumed that rolipram stimulates acid secretion mediated by endogenous histamine released from ECL cells.

PACAP, a biologically active neuropeptide, is also known to be released from the vagus nerves and to stimulate histamine release from ECL cells (15-18). Several studies reported that PACAPs increase gastric acid secretion, but the results are not without controversy. Although PACAP is known to stimulate histamine release from ECL cells (16, 17), this peptide also increases the secretion of somatostatin from D cells, which inhibits acid secretion from parietal cells (20). We also reported that PACAP stimulated gastric acid secretion through the release of histamine from ECL cells, probably mediated by the activation of both PAC1 and VPAC1 receptors, and the acid response to PACAP may be negatively affected by endogenous somatostatin (21). In the present study, PACAP at 10^-7 M caused a minimal increase in acid secretion, the effect being the same as that of histamine at 10^-4 M. This effect was markedly enhanced by rolipram, with a concomitant increase in mucosal cAMP content, and the acid response was totally
inhibited by famotidine. In a preliminary study, we also found that rolipram significantly enhanced the release of histamine in response to PACAP (data now shown). These results suggest that PACAP at 10^{-7} M, though slightly, stimulated acid secretion mediated by endogenous histamine, and rolipram enhanced the acid response to PACAP at two different sites, parietal cells and ECL cells.

Among PDE isoforms PDE1 to PDE5 have been pharmacologically well characterized (10). PDE1 is activated by Ca^{2+}/calmodulin and PDE2 by 3',5'-cyclic guanosine monophosphate (cGMP), yet both of them catalyze the conversion of cAMP and cGMP into inactive metabolites (9). By contrast, PDE3 and PDE4 selectively bind cAMP as a substrate, while PDE5 catalyzes cGMP’s conversion to 5’GMP (11, 12). Although the participation of cAMP in the regulation of acid secretion is well accepted (1, 2, 4), the relation of cGMP to the secretion of acid remains controversial (22-27). Hasebe et al. (27) showed that NO is involved in gastric acid secretion mediated by histamine release from ECL cells. They also showed that both cAMP and cGMP act as second messengers for histamine release from ECL cells, while in the parietal cells these nucleotides have opposite effects on acid secretion; stimulation by cAMP and inhibition by cGMP (25). However, some studies showed an inhibitory effect of NO/cGMP on the process of acid secretion, including histamine release from ECL cells (22-24). In the present study, however, neither vinpocetine (PDE1 inhibitor) nor zaprinast (PDE5 inhibitor) had any effect on acid secretion or histamine release under basal conditions, suggesting that NO/cGMP is not actively involved in the local regulation of acid secretion. Furthermore, since rolipram is an inhibitor of PDE4 that selectively binds to cAMP but not cGMP, it is assumed that the potentiation by this agent of the acid response to histamine or PACAP is brought about by an alteration in cAMP but not cGMP content.

We recently demonstrated in isolated mouse duodenum in vitro that the response of HCO₃⁻ to PGE₂ is regulated by both PDE1 and PDE3, while in the response to NO is modulated by only PDE1 (19). In the stomach, since PGE₂ stimulates HCO₃⁻ secretion mediated by Ca^{2+}, PDE has nothing to do with this process. However, NO stimulated gastric HCO₃⁻ secretion essentially mediated intracellularly by cGMP and modified by both PDE1 and PDE5 (13). We found in the present study that PDE4 is involved in the local regulation of acid secretion. Thus, it is interesting that different PDE isoforms are involved in regulating the same function in different tissues and different functions in the same tissue. In general, the fundamental properties of PDE isoforms are well preserved among species (15). We previously examined the gene expression of PDE isoforms, PDE1–PDE5, including their splicing variants, in the mouse stomach by RT-PCR and confirmed that they were all clearly expressed in this tissue (13). At present, however, since reliable anti-PDE antibodies are not available, it remains unknown which cell type expresses each PDE isoform.

In conclusion, among PDE isoforms, PDE4 is involved in the local regulation of gastric acid secretion via the degradation of cAMP and the PDE4 inhibitor rolipram increases the secretion of acid at two cellular sites; potentiating acid production in parietal cells and enhancing histamine release from ECL cells.

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