# Gastric Acid Secretion in L-Histidine Decarboxylase–Deficient Mice

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*Background & Aims:* **Histamine, gastrin, and acetylcholine are known to be the primary secretagogues of gastric acid secretion, but how the roles are shared among these secretagogues remains to be fully clarified. To evaluate the cooperation between histamine and the other secretagogues, acid secretion responses induced by each secretagogue were measured in L-histidine decarboxylase (HDC)–deficient mice.** *Methods:* **Acid secretion was measured by the titration of acid under anesthesia. The expression of selected genes involved in acid secretion was determined by Northern blot and/or immunoblot analysis. Histamine-2 ($H_2$) receptor binding in the gastric mucosa was investigated using [$^3$H]tiotidine.** *Results:* **HDC-deficient mice showed low basal and high exogenous histamine-stimulated acid secretion. The mutant mice showed hypergastrinemia and did not undergo acid secretion upon treatment with exogenous gastrin. However, carbachol stimulated weak and transient acid secretion in the mutants. The *Bmax* values for $H_2$ and the expression of Gsα in gastric mucosal membranes were higher in the mutants than in the wild-type mice.** *Conclusions:* **This study confirms the concept that histamine production is essential for gastric acid secretion induced by gastrin, but not for that induced by carbachol. HDC-deficient mice should be a suitable model for further functional analyses of the correlation between histamine and the other acid secretagogues.**

The central role of histamine in gastric acid secretion has been studied by many investigators.[1] In 1972, Black et al. reported for the first time that histamine stimulated gastric acid secretion via the histamine-2 ($H_2$) receptor, and then they successfully developed antagonists that were selective for this receptor.[2] These antagonists have potent effects in the treatment of peptic ulcers and the gastroesophageal reflux diseases. Accumulated evidence shows that gastric histamine involved in acid secretion is released from enterochromaffin-like (ECL) cells in rats, and subsequently stimulates the $H_2$ receptors on the parietal cells.[3,4] In addition to histamine, gastric acid secretion is regulated by a complex set of mechanisms involving the neurocrine, endocrine, paracrine, and autocrine pathways.[1] Regarding the peripheral regulation of gastric acid secretion, it is now considered that the major stimuli acting on the parietal cells are histamine, acetylcholine, and gastrin. Gastrin has been reported to stimulate acid secretion in 2 ways: (1) by acting on ECL cells to stimulate histamine release and the de novo formation of histamine, and (2) by acting directly on parietal cells to stimulate acid secretion.[5,6] Acetylcholine has been found to stimulate acid secretion in 2 ways: (1) by the activation of the muscarinic-3 ($M_3$) receptors on parietal cells, and (2) by stimulating histamine release from ECL cells via the activation of the $M_1$ receptors.[7,8] It must be noted, however, that the interaction between these secretagogues in acid secretion is complex and not fully understood. It has been difficult to evaluate the function of one of the secretagogues separately from that of another, because one secretagogue often induces another and because the efficacy of antagonists is incomplete and does not last for long periods of time. Mutant mice that genetically lack a specific receptor or enzyme represent ideal models for such analyses. Recently, gastrin-deficient and gastrin/cholecystokinin (CCK)-B receptor–deficient mouse strains were generated and their phenotypes with respect to gastric acid

*Abbreviations used in this paper:* ECL, enterochromaffin-like; $H_2$, histamine-2; HDC, histidine decarboxylase; $M_3$, muscarinic-3; PGK, phosphoglycerate kinase; PMSF, phenylmethylsulfonyl fluoride; RT-PCR, reverse-transcription polymerase chain reaction; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline.


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secretion and mucosal cellular compartments were examined.[9–12] In gastrin-deficient mice, the induction of acid secretion was undetectable after stimulation with any of the secretagogues that were examined, which suggests a fundamental requirement for gastrin in acid secretion.[9] Histochemical studies and the analyses of parietal and ECL cell marker expression showed some defects in both cell types in the gastrin-deficient mice.[10] Because the histamine content and the expression of histidine decarboxylase (HDC) messenger RNA in gastric mucosa was markedly reduced in the gastrin-deficient mice, the parietal cell defect may be caused by a reduction in histamine, in addition to the absence of gastrin.[9] Because gastrin- and gastrin/CCK-B receptor–deficient mice had defects in the acid secretory machinery, the function of histamine and its cooperation with gastrin in acid secretion could not be fully clarified in studies with these mice.

We recently generated an HDC-deficient mouse strain by gene targeting in embryonic stem cells.[13] HDC has been considered to be the only enzyme that forms histamine in mammals. Consistently, our mutant mice exhibited extremely low levels of tissue histamine content, and the de novo synthesis of histamine was undetectable in these mice. We have analyzed the effects of exogenously administrated histamine, gastrin, and carbachol on gastric acid secretion in these HDC-deficient mice.

## Materials and Methods

### HDC-Deficient Mice

HDC-deficient mice were generated as described elsewhere.[13] We designed the HDC targeting construct to replace a ~2.4-kb fragment extending from intron 5 to exon 9 with a phosphoglycerate kinase (*PGK*) promoter-*neo*$^r$ cassette. We then replaced exons 6–8 with an inverted PGK promoter-driven neomycin phosphotransferase (*neo*) gene. This replacement should result in a loss-of-function mutation because exon 8 contains the coding sequence for the putative binding site (TFNPSKW) for pyridoxal 5′-phosphate, the coenzyme of the HDC protein.[14] Eight to 12-week-old wild-type and HDC$^{-/-}$ mice, bred and maintained independently, were used in all experiments. Mice were fasted for 24 hours before each experiment with free access to tap water. The HDC-deficient mice developed without any gross abnormalities and exhibited normal weight gain and fertility in comparison with the wild-type mice.

### Histidine Decarboxylase Assay

The stomach of each mouse was minced, suspended in lysis buffer (10 mmol/L HEPES, pH 7.3, containing 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol, 1% Triton X-100, 1 mmol/L EDTA, 1 mmol/L ethylene glycol bis [β-aminoethyl ether]-*N,N,N′,N′*-tetraacetic acid, 0.2 mmol/L phenylmethylsulfonyl fluoride [PMSF], 0.1 mmol/L benzamidine, 10 μg/mL leupeptin, 10 μg/mL aprotinin, 10 μg/mL E-64, and 1 μg/mL pepstatin A), and homogenized by 40 strokes with a Teflon/glass homogenizer. The homogenate was centrifuged at 1000*g* for 10 minutes at 4°C to remove the debris. The supernatant was recentrifuged at 100,000*g* for 1 hour at 4°C. The resultant supernatant was assayed for HDC activity as described previously.[15] Briefly, the assay mixture (1 mL) was comprised of 0.8 μmol L-histidine, 0.2 μmol dithiothreitol, 0.01 μmol pyridoxal 5′-phosphate, 10 mg polyethylene glycol #300, 100 μmol potassium phosphate (pH 6.8), 0.2 μmol aminoguanidine, and enzyme. The reaction was carried out at 37°C for 4 hours and was terminated by adding 0.05 mL of 60% perchloric acid. The histamine formed was separated on a WCX-1 cation exchange column (Shimadzu, Kyoto, Japan) by high performance liquid chromatography and then measured by the *o*-phtalaldehyde method.[16]

### Protein Assay

Protein concentrations were determined by the method of Bradford using bovine serum albumin for the standards.[17]

### Immunohistochemical Analysis

Stomachs were collected from wild-type and mutant mice that had been fasted for 24 hours and were treated with Carnoy's fixative (Muto Pure Chemicals, Tokyo, Japan) for 14 hours at 4°C and then paraffin-embedded. Sections 4 μm in thickness were cut and deparaffinized in xylene. The sections were immersed in 0.3% hydrogen peroxide in methanol for 30 minutes to block endogenous peroxidase activity. The sections were incubated with 1% normal goat serum at 4°C for 1 hour for blocking, followed by incubation with an anti $H^+$, $K^+$-adenosine triphosphatase (ATPase) α subunit antibody (1:200) (MBL, Nagoya, Japan)[18] or with an anti-chromogranin A antibody (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA)[19] for 14 hours at 4°C. After incubation with a biotinylated secondary antibody against immunoglobulin G (1:200; Vector, Burlingame, CA) for 2 hours, the antibodies were detected with the avidin-biotin-peroxidase complex (diluted 1:200; Vector). Development was performed by incubation with 50 mmol/L Tris-HCl, pH 7.6, containing 0.02% 3,3′-diaminobenzidine, 0.0045% $H_2O_2$, and 0.6% nickel ammonium sulfate for 3 minutes to obtain brown-stained products. The sections were counterstained with hematoxylin. H&E staining was performed to obtain a general appearance of the sections.

### Northern Blot Analysis

Stomachs were collected from each mouse and immediately frozen in liquid nitrogen and stored at −80°C until use. Total RNA was extracted by the acid guanidium thiocyanate-phenol-chloroform method.[20] Poly($A^+$) RNA was further purified from total RNA by oligo (dT) Latex (TaKaRa, Kyoto, Japan). Poly($A^+$) RNA (5 μg) was separated by electrophoresis on a 1.5% agarose gel and transferred onto a nylon

membrane (Biodyne-A, Pall, Port Washington, NY). Hybridization was performed with a [$^{32}$P]-labeled complementary DNA (cDNA) fragment specific for the genes indicated below at 68°C in 6 × standard saline citrate, 0.5% sodium dodecyl sulfate (SDS), and 5 × Denhardt's solution. After hybridization, filters were washed at 68°C in 2 × standard saline citrate and 1% SDS, and the hybridized bands were detected by autoradiography. The filters were then rehybridized with a [$^{32}$P]-labeled cDNA fragment specific for glyceraldehyde-3-phosphate dehydrogenase (CLONTECH, Palo Alto, CA). The template cDNAs for the other genes were generated by PCR amplification and subcloned using the specific primers listed below. The mouse $H_2$ receptor probe (747 base pairs) was reverse-transcription polymerase chain reaction (RT-PCR) amplified with primers 5′-ACG GAT CCG TCA ACG AGG TAT ATG GAC TGG-3′ and 5′-ACG AGC TCC TCT GGA GTG AGT GAG TAA CAT TCT-3′. The mouse $M_3$ receptor probe (630 base pairs) was RT-PCR amplified with primers 5′-ACG GAT CCC AGA GAG TCA CAA TGA CCT TGC-3′ and 5′-ACG AGC TCG CCA GAA CAA GAT GGC AGG AG-3′. The gastrin/CCK-B receptor probe (767 base pairs) was RT-PCR amplified with primers 5′-ACG GAT CCT GTC CAC TCT AAA TCT CGC GGC CAT C-3′ and 5′-ACG AGC TCC GGA ATC GGC GGT GCA TGA AAC AGT A-3′. The gastrin probe (469 base pairs) was RT-PCR amplified with primers 5′-ACG GAT CCG TAC ATG CTG GTC TTA GTG CTG GCT C-3′ and 5′-ACG AGC TCT GTT TTG TAA GGA CGG AGC TGG GGG A-3′. The $H^+$, $K^+$-ATPase α subunit probe (877 base pairs) was RT-PCR amplified with primers 5′-ACG GAT CCA AGG AGA AGC TGG AGA ACA TGA AGA AGG-3′ and 5′-ACG AGC TCA TGG CCC GAA GGA AGG TAT AGC CAA T-3′. RT-PCR–amplified products were verified by nucleotide sequence analysis.

## Gastric Acid Secretion

Each mouse was fasted for 24 hours, with free access to tap water, and anesthetized with an intraperitoneal injection of urethane at 1.25 g/kg. After tracheotomy, a polyethylene tube was inserted into the trachea to ensure a patent airway. The abdomen was incised and the stomach and duodenum were exposed. An acute fistula was made by the insertion of a polyethylene tube toward the antrum through an incision of the duodenum. Four hundred microliters of saline was injected and collected every 15 minutes, and acid output (μmol $H^+$/15 min) was determined by titration with 10 mmol/L NaOH. Mice were injected with 100 μL of each reagent solution subcutaneously. Drugs were prepared as follows: histamine dihydrochloride, [Leu$^{15}$] human gastrin I, carbachol and atropine in saline, famotidine in 0.1% carboxymethyl cellulose, and YF-476 and YM-022 in polyethylene glycol #300. Control animals were injected with vehicle solution. Although it has been reported that polyethylene glycol causes suppression of the acid output in rats,[21] we used this solvent because of the insolubility of the gastrin/CCK-B antagonists into aqueous solution.

## Scatchard Analysis of [$^3$H]Tiotidine Binding

Stomachs were collected from mice fasted for 24 hours, minced, and homogenized by 20 strokes with a Teflon/glass homogenizer in homogenization buffer (10 mmol/L sodium phosphate, pH 7.4, containing 0.25 mol/L sucrose, 10 mmol/L $MgCl_2$, 1 mmol/L EDTA, 1 mmol/L dithiothreitol, and 0.1 mmol/L PMSF). The homogenate was centrifuged at 1000*g* for 10 minutes at 4°C. The supernatant was recentrifuged at 100,000*g* for 1 hour at 4°C. The resultant precipitate fraction (designated as gastric mucosal membranes) was resuspended in binding buffer (150 mmol/L sodium phosphate, pH 7.4). One hundred microliters of membranes containing 100 μg protein was incubated in triplicate with various concentrations (2–24 nmol/L) of [$^3$H]tiotidine (~90 Ci/mmol; New England Nuclear, Boston, MA) in the presence or absence of 0.1 μmol/L unlabeled tiotidine (for the determination of nonspecific binding). Incubations were carried out at 4°C for 40 minutes and terminated by dilution with 2 mL of ice-cold binding buffer.[22] The mixture was immediately filtrated under reduced pressure onto Whatman GF/B glass-fiber filters followed by 2 washes with ice-cold binding buffer. The filters were dried and their radioactivities determined by liquid scintillation counting.

## Assay for Gastrin Peptides

The serum of each mouse was collected and the gastrin peptide content was determined by a radioimmunoassay (Mitsubishi Kagaku Bio-Clinical Laboratories, Inc., Tokyo, Japan).

## Immunoblot Analysis

The stomach of each mouse was collected and homogenized by 20 strokes with a Teflon/glass homogenizer in 25 mmol/L sodium phosphate, pH 7.4, containing 10 mmol/L HEPES-NaOH, pH 7.3, 5 mmol/L $MgCl_2$, and 0.1 mmol/L PMSF. The homogenates were further homogenized with a Polytron homogenizer and centrifuged at 800*g* for 15 minutes at 4°C. The supernatant was recentrifuged at 30,000*g* for 30 minutes at 4°C. The resultant precipitate was resuspended in the same buffer. The aliquot containing 50 μg of membrane proteins was boiled for 15 minutes in SDS sample buffer (62.5 mmol/L Tris-HCl, pH 6.8, containing 2% SDS, 10% glycerol, 0.01% bromophenol blue, and 5% 2-mercaptoethanol). SDS–polyacrylamide gel electrophoresis was performed on slab gels (10%). Protein samples were subjected to SDS–polyacrylamide gel electrophoresis, and the separated proteins were transferred electrophoretically onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA) in 25 mmol/L Tris base containing 40 mmol/L 6-aminohexanoic acid, 0.02% SDS, and 20% methanol at room temperature for 45 minutes at 15 V. The membrane was rinsed in Tris-buffered saline (TBS; 20 mmol/L Tris-HCl, pH 7.5, containing 150 mmol/L NaCl) and then preincubated overnight in TBS containing 5% non-fat milk at 4°C. The membrane was then incubated with an anti-$H^+$, $K^+$-ATPase α subunit antibody (1:200), an anti-chromogranin A antibody (1:200), an anti-Gsα antibody (1:200) (Santa Cruz Biotechnology, Santa Cruz, CA),[23] or an anti-Giα antibody (1:1000) (NEN Research Products, Boston, MA)[24] in TBS

containing 5% non-fat milk for 1 hour at 37°C. The membrane was washed 3 times with TBS containing 0.05% Tween 20 (TTBS) at room temperature, then incubated with an anti-mouse (goat or rabbit) immunoglobulin G antibody conjugated with horse radish peroxidase (DAKO, Glostrup, Denmark) in TTBS for 1 hour at room temperature, and then stained with an ECL staining kit (Amersham Pharmacia, Uppsala, Sweden).

### Materials

YM022 and YF476 were kindly provided by Yamanouchi Pharmaceutical Industries, Ltd. (Tokyo, Japan). The following materials were purchased from the sources indicated: histamine dihydrochloride from Wako Pure Chemicals (Osaka, Japan), famotidine, [Leu$^{15}$] human gastrin I and atropine from Sigma (St. Louis, MO), carbachol from CARBIOCHEM (San Diego, CA), and tiotidine from TOCRIS (Bristol, England). All other chemicals were commercial products of reagent grade.

## Results

### General Appearance of HDC-Deficient Mice

The murine *HDC* gene contains 12 exons and spans 23 kilobases of genomic DNA.[25] Using gene targeting, we replaced exons 6–8 with an inverted PGK promoter-driven neomycin phosphotransferase (*neo*) gene. Homologous recombination was confirmed by Southern blot analysis and genomic PCR (Ohtsu et al.[13] and data not shown). Northern blot analysis showed the absence of HDC transcripts (2.7 kilobases) in the stomachs of the HDC-deficient mice (data not shown). HDC-deficient mice showed no HDC activity and a negligible amount of histamine (<50 ng/g tissue) in their gastric mucous homogenates. The level of basal acid secretion in the mutants was slightly lower than in the wild-type mice (Table 1). Although the mutants showed pronounced hypergastrinemia (Table 1), no significant changes were observed in the general histologic appearance of the gastric mucosal cells (Figure 1*A* and *B*). Immunohistochemical staining using an anti-$H^+$, $K^+$-ATPase α subunit antibody or an anti–chromogranin A antibody showed similar distributions of parietal and ECL cells in the gastric fundic region of the wild-type and mutant mice (Figure 1*C–F*). No significant changes in the number of parietal and ECL cells were observed between the wild-type and mutant mice (data not shown).

**Table 1.** General Appearance of HDC-Deficient Mice

| Genotype | +/+ | −/− |
|---|---|---|
| HDC activity (*pmol · min$^{-1}$ · mg protein$^{-1}$*) | 1.30 ± 0.357 (n = 4) | N.D. (n = 4) |
| Histamine content (*μg/g tissue*) | 12.4 ± 1.42 (n = 4) | N.D. (n = 4) |
| Serum gastrin (*pg/mL*) | 100 ± 24.5 (n = 4) | 302 ± 31.2[a] (n = 4) |
| Basal acid secretion (*μmol $H^+$/15 min*) | 0.59 ± 0.02 (n = 38) | 0.52 ± 0.01[a] (n = 45) |

NOTE. Each parameter was measured in the wild-type (+/+) and the mutant (−/−) mice as described in Materials and Methods. Values obtained are represented as the means ± SEM.
N.D., not detected.
[a]$P < 0.01$, by Alternate Welch *t* test.

### Gastric Acid Secretion Stimulated by Histamine

Gastric acid secretion responses of the mutants were compared with those of the wild-type mice using various doses of histamine in the presence or absence of famotidine (Figure 2). In the wild-type mice, a dose of 3 mg/kg histamine did not induce significant acid secretion, whereas 10 mg/kg histamine markedly increased acid secretion, resulting in a maximal response. On the other hand, acid secretion in the mutants was significantly induced by treatment with 1 mg/kg histamine. The maximal acid output in the mutant mice treated with 3 mg/kg histamine was much higher than the maximal secretion observed in the wild-type mice. Maximal acid output was about 2-fold higher in the mutants compared with the wild-type mice. Histamine-stimulated acid secretion was completely abolished in both the wild-type mice and the mutants by pretreatment with 10 mg/kg famotidine for 1 hour before the stimulation. Serum histamine concentrations were significantly elevated 30 minutes after the subcutaneous injection, although no significant differences were observed in the concentration of histamine between the wild-type and mutant mice (Table 2). Furthermore, serum gastrin concentrations in the wild-type mice remained unchanged on the injection of histamine, whereas those in the mutant mice were decreased by about 50%.

### Gastric Acid Secretion in Response to Gastrin

A dose of 1 mg/kg of [Leu$^{15}$] human gastrin I stimulated acid secretion in the wild-type mice. Acid secretion reached maximal levels 30 minutes after the stimulation and was sustained thereafter; furthermore, acid secretion was completely inhibited by pretreatment with famotidine. In contrast, [Leu$^{15}$] human gastrin I could not stimulate acid secretion in the mutants, even at a dose of 3 mg/kg (Figure 3). The acid secretion responses induced by [Leu$^{15}$] human gastrin I in the wild-type mice were abolished by the gastrin/CCK-B receptor antagonists, YM022 and YF476 (10 μmol/kg; data not shown).

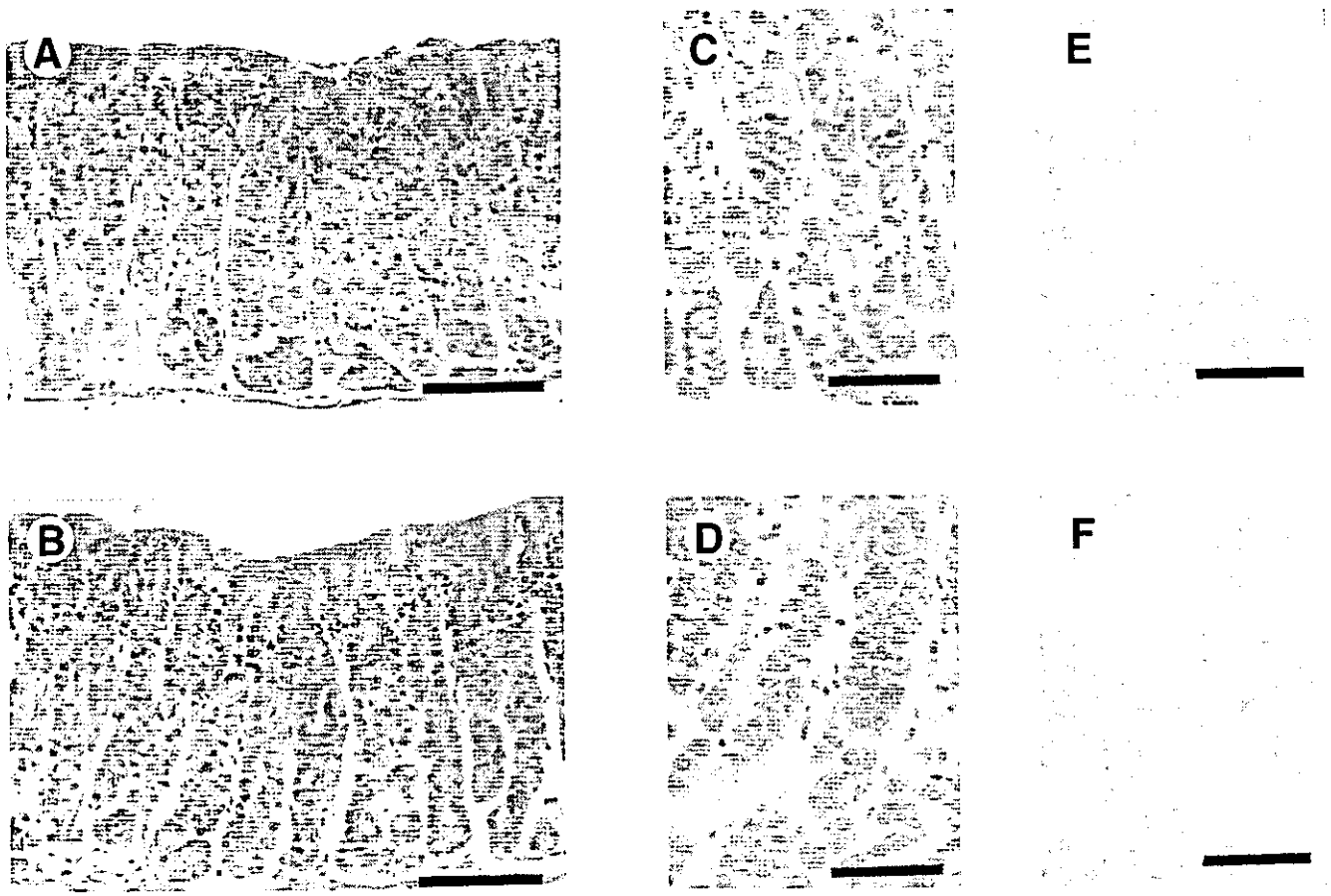


**Figure 1.** Histologies of the gastric mucosa in the fundic region of (*A*, *C*, and *E*) wild-type and (*B*, *D*, and *F*) mutant mice. (*A* and *B*) H&E-stained sections (4 μm in thickness). (*C–F*) Immunohistochemical staining of parietal cells and ECL cells (*C* and *D*) with an antibody against the $H^+$, $K^+$-ATPase α subunit (1:200) or (*E* and *F*) with an antibody against chromogranin A (1:500). (*A* and *B*, *bar* = 100 μm; *C–F*, *bar* = 50 μm.)

### Gastric Acid Secretion in Response to Carbachol

Treatment with 0.1 mg/kg carbachol was found to induce a biphasic acid secretion response in the wild-type mice. The early phase (~30 minutes) was characterized by famotidine-insensitive secretion, whereas the late phase was characterized by famotidine-sensitive secretion (Figure 4). Both phases were completely abolished by pretreatment with 3 mg/kg of atropine. In contrast, when the mutant mice were treated with carbachol, acid secretion was induced only in the early phase, and not in the late phase.

### Effect of Atropine or Gastrin/CCK-B Antagonists on Histamine-Induced Acid Secretion

In both the wild-type and the mutant mice, acid secretion induced by 10 mg/kg of histamine was reduced by pretreatment with 3 mg/kg atropine (Figure 5). The degree of inhibition in the wild-type mice was about 55% in cumulative acid output, whereas that in the mutants was about 60%. Histamine-induced acid secretion in the mutants treated with atropine remained higher than that in the wild-type mice but was not significant when calculated as cumulative acid output (Figure 5). Subcutaneous injection of YF476 (10 μmol/kg) resulted in a considerable inhibition of acid secretion induced by histamine in the wild-type mice, whereas it did not suppress the histamine-induced acid secretion in the mutants (Figure 6). Treatment with YM022 (10 μmol/kg) demonstrated the same tendencies as that with YF476 (data not shown).

### Expression of Genes and Proteins Involved in Gastric Acid Secretion

In both gastrin- and gastrin/CCK-B receptor-deficient mice, a drastic alteration in the expression of genes involved in gastric acid secretion was observed.[9,12] We investigated the expression levels of the following genes in the mutant mice: the $H_2$, $M_3$, and gastrin/CCK-B receptors, gastrin, and the $H^+$, $K^+$-ATPase α subunit. The expression levels of all these genes in the mutants were comparable to those in the wild-type mice (Figure 7*A*). Similarly, the expression of the $H^+$, $K^+$-

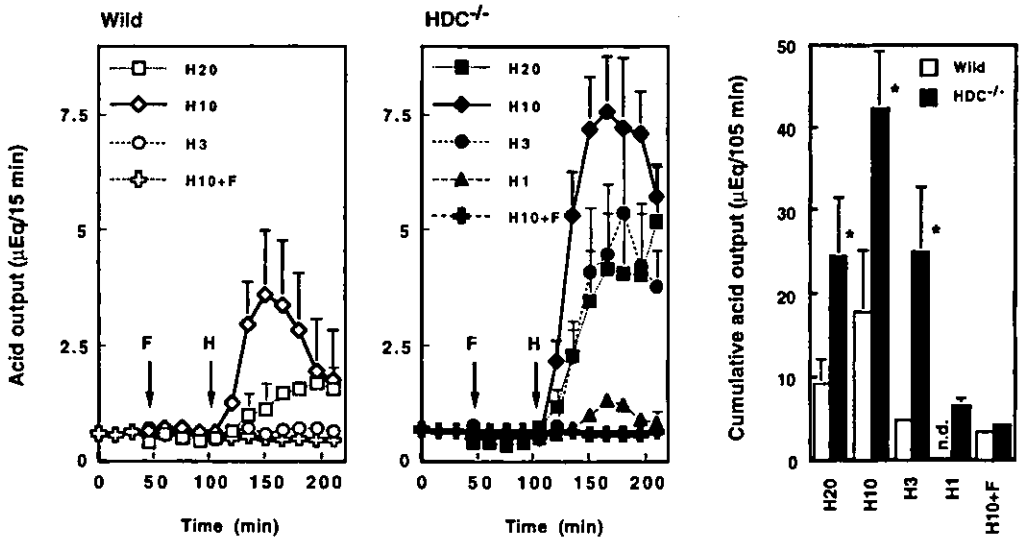


**Figure 2.** Gastric acid output stimulated by exogenously administrated histamine in wild-type and mutant mice. Histamine was injected subcutaneously at a dosage of 1 mg/kg (H1), 3 mg/kg (H3), 10 mg/kg with (H10+F) or without (H10) pretreatment with famotidine (10 mg/kg subcutaneously), or 20 mg/kg (H20). The profiles of acid output in the wild-type (Wild) (H3, n = 5; H10, n = 6; H10+F, n = 3; H20, n = 3) and the mutant mice ($HDC^{-/-}$) (H1, n = 3; H3, n = 5; H10, n = 8; H10+F, n = 3; H20, n = 3) are represented as the means ± SEM. The cumulative acid output after each stimulation is represented in the *right panel*. Statistical differences were evaluated using the Student *t* test; $*P < 0.05$ was regarded as significant; n.d., not determined.

ATPase α subunit and chromogranin A was unchanged in the mutants at the level of protein expression (Figure 7*B*). Nevertheless, the expression of Gsα but not that of Giα in the gastric membranes was significantly increased in the mutants (Figure 7*B*).

### Binding Analysis for Gastric $H_2$ Receptors

Scatchard plot analyses demonstrated a lower binding affinity and a higher binding maximum for [$^3$H]tiotidine, a specific $H_2$ antagonist in the gastric membrane fraction of the HDC-deficient mice (Figure 8). *Kd* and *Bmax* values were as follows: *Kd*, 3.24 (wild-type mice) vs. 17.5 nmol/L (mutant mice); *Bmax*, 1.09 (wild-type mice) vs. 1.88 pmol/mg protein (mutant mice).

**Table 2.** Serum Concentrations of Gastrin and Histamine After Histamine Injection

| | Gastrin (*pg/mL*) | Histamine (*μg/mL*) |
|---|---|---|
| Wild-type (control) | 43.8 ± 4.19 | 0.138 ± 0.00291 |
| Wild-type (+ histamine) | 52.5 ± 8.05 | 1.59 ± 0.347[a] |
| $HDC^{-/-}$ (control) | 269 ± 36.7 | N.D. |
| $HDC^{-/-}$ (+ histamine) | 123 ± 18.1[a] | 1.67 ± 0.182 |

NOTE. Serum was collected from mice 30 minutes after injection with saline (control) or 10 mg/kg histamine (+ histamine) under urethane anesthesia. Each parameter was measured in the wild-type and the mutant ($HDC^{-/-}$) mice as described in Materials and Methods. Values obtained are represented as the means ± SEM (n = 4).
N.D., not detected.
[a]$P < 0.05$, control vs. + histamine, by the Student *t* test.

## Discussion

This study showed that HDC-deficient mice acquire a high sensitivity to exogenous histamine regarding gastric acid secretion. After a 24-hour fasting, the mutant mice were much more susceptible to low

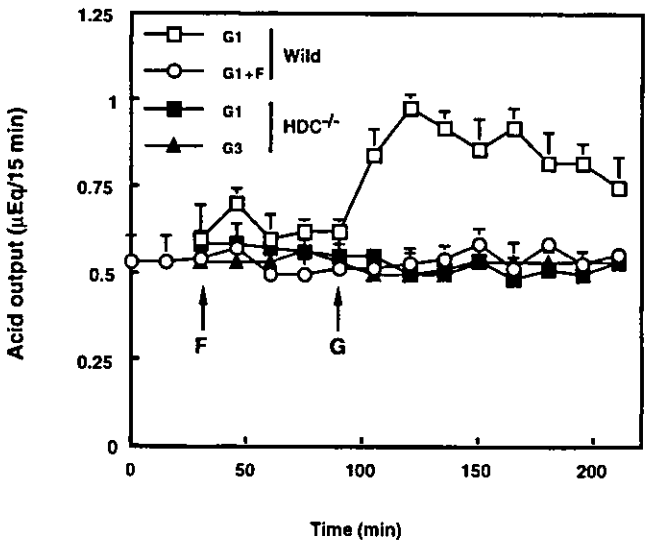


**Figure 3.** Gastric acid output stimulated by exogenously administrated [Leu$^{15}$] human gastrin I in wild-type and mutant mice. The wild-type mice were injected subcutaneously with 1 mg/kg of [Leu$^{15}$] human gastrin I (G1, n = 5), whereas the mutant mice were injected with 1 mg/kg (G1, n = 8) or 3 mg/kg human gastrin I (G3, n = 3). Gastric acid outputs are represented as the means ± SEM. Pretreatment with famotidine (10 mg/kg, subcutaneously) was performed in the wild-type mice (G1+F, n = 3).

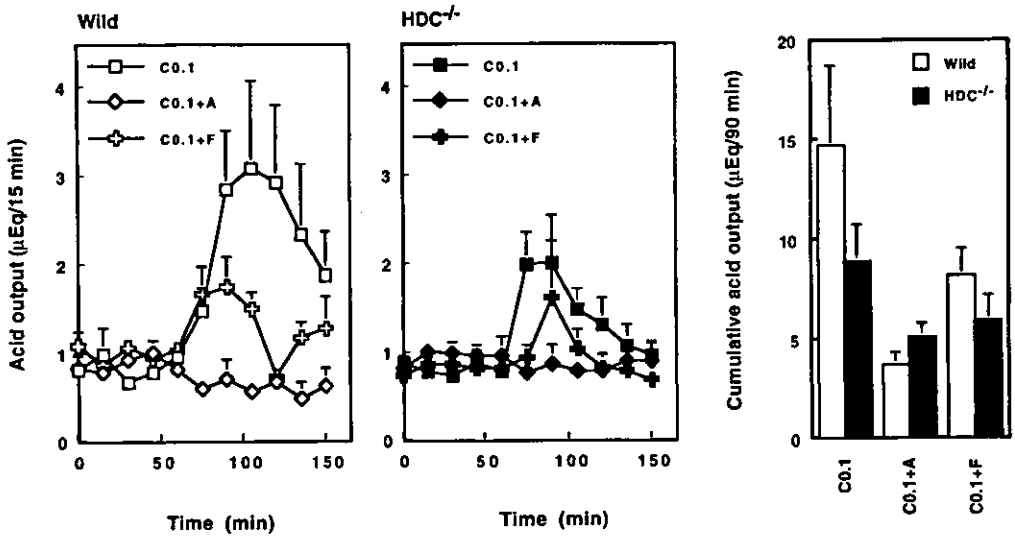


**Figure 4.** Gastric acid output stimulated by carbachol in wild-type and mutant mice. The wild-type mice (Wild: C0.1, n = 7) and the mutant mice ($HDC^{-/-}$: C0.1, n = 7) were injected subcutaneously with 0.1 mg/kg carbachol. Gastric acid outputs were represented as the means ± SEM. For blockade of the $H_2$ or muscarinic receptors, pretreatment with famotidine (C0.1+F; 10 mg/kg subcutaneously; n = 3) or with atropine (C0.1+A; 3 mg/kg subcutaneously; n = 3), respectively, was performed in the wild-type and the mutant mice. The cumulative acid output after each stimulation is represented in the *right panel*. No statistical differences were obtained in the values of the wild-type mice and that of the mutants using the Student *t* test.

doses of exogenous histamine and showed a high plateau level of gastric acid secretion. This response seems to result exclusively from an $H_2$ receptor–mediated reaction in the parietal cells because it was completely suppressed by famotidine. Basal acid secretion was maintained at a slightly lower level than wild-type in the mutants. Because the mutants maintained their responses to carbachol, but not to gastrin, basal acid secretion seems to be partially compensated for by the muscarinic pathway. We observed a trace amount of histamine in various tissues as a result of daily ingestion upon consumption of a regular diet (Ohtsu H and Tanaka S, unpublished observation, June, 1999). Subsequently, we were not able to exclude the possibility that a very low level of tissue histamine may have a role in the maintenance of basal

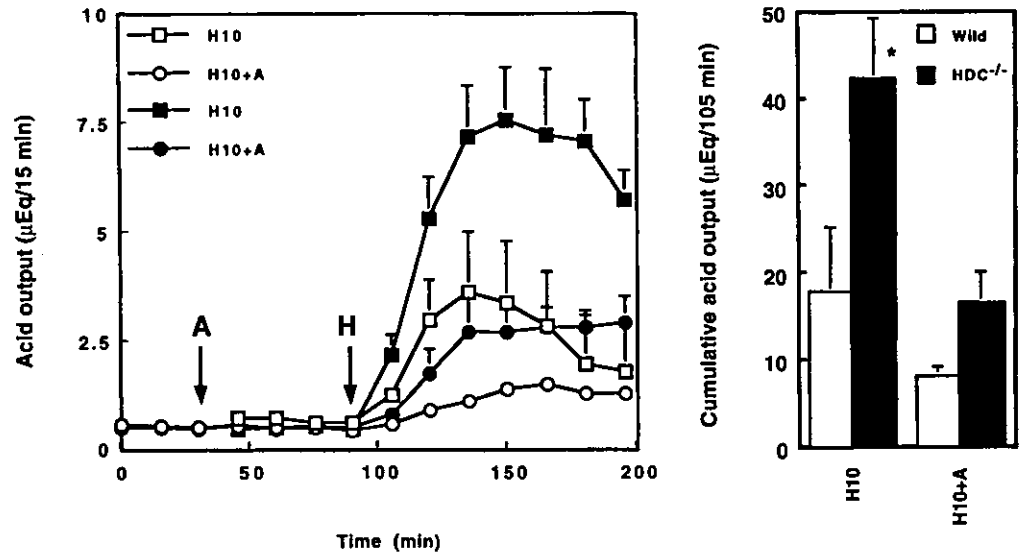


**Figure 5.** The inhibitory effects of atropine on histamine-stimulated acid secretion in wild-type and mutant mice. The wild-type mice (○, H10+A; n = 5) and the mutants (●, H10+A; n = 8) were injected subcutaneously with 3 mg/kg atropine 1 hour before stimulation with histamine (10 mg/kg subcutaneously). Gastric acid outputs are represented as the means ± SEM. This experiment was performed at the same time with that of Figure 2, and a part of the acid output profiles from Figure 2 are overlaid for comparison (□, wild-type mice; ■, mutants; H10). The cumulative acid output for each treatment is represented in the *right panel* (*$P < 0.05$).

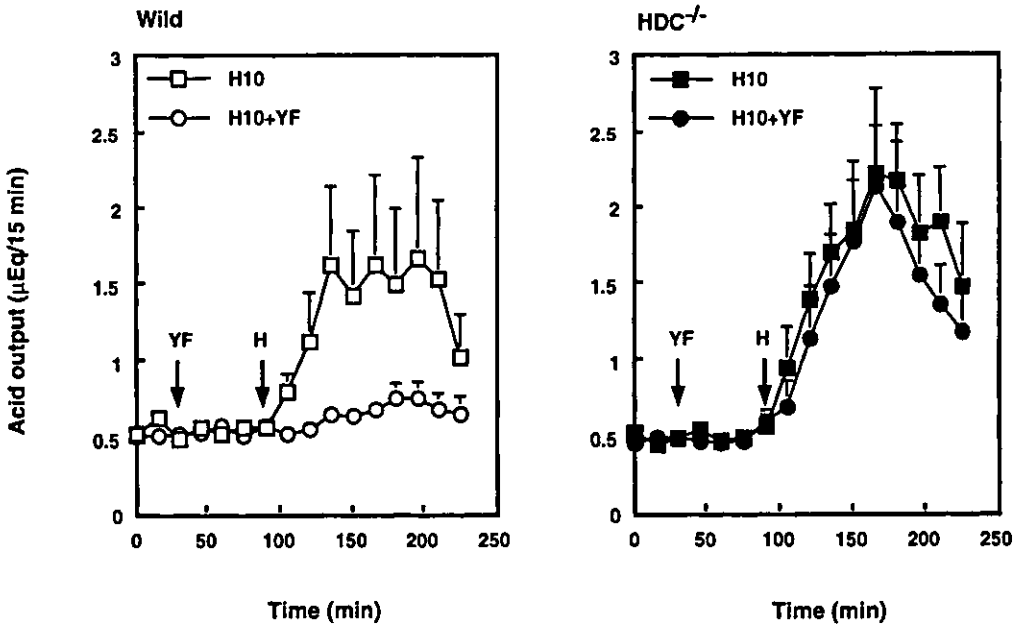


**Figure 6.** The effect of YF476, a gastrin/CCK-B antagonist, on histamine-stimulated acid secretion in the wild-type and the mutant mice. The wild-type mice (Wild) and the mutants ($HDC^{-/-}$) were injected subcutaneously with 10 mg/kg histamine with (H10+YF; n = 3) or without (H10; wild-type mice, n = 5; mutants, n = 8) 10 μmol/kg YF-476 in polyethylene glycol #300. Gastric acid outputs are represented as the means ± SEM.

gastric acid secretion in the mutant mice, although the gastric histamine content in the mutant mice was undetectable.

It has been reported that cholinergic stimulation of acid secretion occurs via 2 distinct pathways. One pathway is histamine-dependent and has an indirect effect on the parietal cells, whereas the other pathway is histamine-independent and has a direct effect on the parietal cells.[26] Sandor et al. have reported that carbachol stimulated both the basal and gastrin-driven release of histamine from isolated ECL cells, probably by the activation of $M_1$ receptors.[8] On the other hand, Soll et al. have reported that in isolated canine parietal cells, carbachol potently stimulated aminopyrine uptake via the $M_3$ receptors.[27] Such current evidences suggest that muscarinic stimulation may act both directly on parietal cells to stimulate acid secretion and indirectly via the release of histamine from ECL cells. The relative importance of these 2 modes of action in vivo is not clear. Cholinergic stimulation of acid secretion in isolated gastric glands has been reported to be weak and transient in the presence of $H_2$ antagonists.[28] The residual activity of cholinergic stimulation that is not inhibited by $H_2$ antagonists has been shown to be blocked by atropine.[27] The results of the present study support these previous findings. A rapid increase in acid secretion induced by carbachol was observed in both the wild-type and mutant mice, indicating that carbachol acts directly on the $M_3$ receptors on parietal cells. The sustained phase in acid secretion, which is sensitive to famotidine, was observed only in the wild-type mice, indicating that the activation of the $M_1$ receptors on ECL cells may stimulate histamine release. Atropin pretreatment decreased acid secretion in both the wild-type and the mutant mice. In the presence of atropine, histamine-induced acid secretion in the mutants was no longer significantly higher than in the wild-type mice. These observations indicate that under urethane anesthesia, cholinergic effects should have some contribution to the enhancement of histamine-induced acid secretion in the mutant mice.

It is generally accepted that gastrin acts to induce gastric acid secretion via 2 different pathways. One pathway represents the stimulation of histamine release from ECL cells, and the other pathway represents the direct activation of parietal cells. Our results showed that neither a high level of endogenous gastrin nor the addition of [$Leu^{15}$] human gastrin I was able to induce gastric acid secretion in the mutant mice. This indicates that gastrin-induced acid secretion is solely mediated by endogenous histamine formation in ECL cells. It is likely that the activation of gastrin/CCK-B receptors on parietal cells is unable to induce acid secretion in the absence of histamine.

To our surprise, the gastrin/CCK-B antagonists, YF476 and YM022, significantly inhibited acid secretion induced by histamine in the wild-type mice, although both antagonists were found to exhibit no inhibitory effect on histamine-induced acid secretion in rats and dogs.[29,30] Bliss et al. have reported that histamine promotes gastrin release from isolated rabbit G cells via the $H_2$ receptors.[31] In our experiments, however, exogenous histamine did not elevate serum gastrin concentrations in the wild-type mice and rather decreased those in the mutant mice. This observation excludes the possibil-

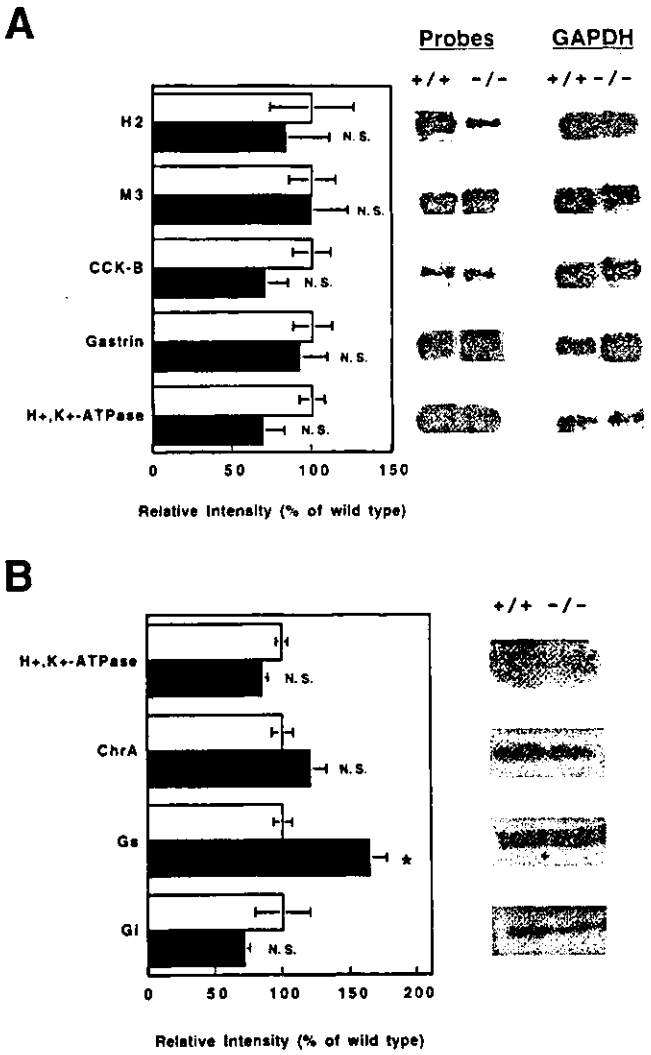


**Figure 7.** The expression of genes and proteins involved in gastric acid secretion in wild-type and mutant mice. (*A*) Poly $A^+$ RNA (5 μg/well) was separated on a 1.5% agarose gel and transferred onto a nylon membrane. The probe for glyceraldehyde-3-phosphate dehydrogenase was used as a loading control. The relative intensity of each band was calculated and represented as a percentage of the mean intensity in the wild-type mice (□, wild-type mice, n = 4; ■, mutants, n = 4). The data are represented as the means ± SEM. N.S., not significant ($P > 0.05$). (*B*) Immunoblot analyses with an anti-$H^+$, $K^+$-ATPase α subunit, anti-chromogranin A (Chr A), anti-Gsα, and anti-Giα antibody were performed (50 μg protein/each lane). The relative intensity of each band was determined densitometrically using the NIH Image version 1.55 software and represented as the means ± SEM. n = 4. *$P < 0.05$. N.S., not significant, $P > 0.05$.

ity that exogenous histamine may induce endogenous gastrin release. The inhibitory effects of the gastrin/CCK-B antagonists on histamine-induced acid secretion in the wild-type mice indicate that gastrin/CCK-B receptor signaling is required for acid secretion induced by histamine. Our observation suggests that gastrin and histamine may be equally indispensable in the mouse gastric acid secretion system. Our observation that the gastrin/CCK-B antagonists did not suppress acid secretion induced by histamine in the mutants is, however, inconsistent with this hypothesis. Although we are not able to resolve this inconsistency from the current results, one possible explanation is that acid secretion in the mutants may be compensated for by factors other than gastrin and may differ in the mechanism from that in the wild-type mice. Further investigations are required, such as studies on the signal cross-talk between $H_2$ and gastrin/CCK-B receptors in primary cultures of parietal cells, to clarify the mechanism by which gastrin and histamine induces acid secretion cooperatively. It has been difficult to separate the role of gastrin from that of histamine in acid secretion because the activation of ECL cells by gastrin is always accompanied by histamine release. In the studies using gastrin- or gastrin/CCK-B receptor–deficient mice, it remains to be determined whether the defects in acid secretion in these mice are due to the loss of direct gastrin effects or to a decrease in gastrin-mediated histamine release and/or synthesis, because histamine function is considered to be compromised in these models.[9,12]

Gastrin/CCK-B receptor–mediated signal transduction is also considered to be essential for the normal development of the gastric mucosa.[9–12] Furthermore, elevated gastrin levels have been found to enhance the development of parietal and ECL cells, as well as ECL carcinoid formation.[32–34] We were, however, unable to observe parietal and ECL cell hyperplasia in the mutant mice, although they exhibited hypergastrinemia. $H_2$ re-

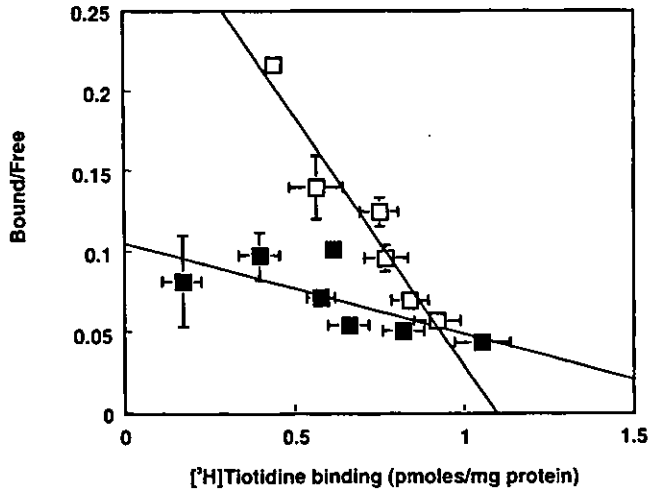


**Figure 8.** $H_2$ binding study of the membrane fraction of the gastric mucosa from wild-type and mutant mice. Gastric mucosal membranes (100 μg protein) from wild-type (□) or mutant (■) mice were incubated in triplicate for 40 minutes at 4°C with various concentrations (2–24 nmol/L) of [$^3$H]tiotidine, respectively. The radioactivities were determined by liquid scintillation counting. Each value obtained from Scatchard analysis was represented as the means ± SEM.

ceptor–deficient mice recently have been developed by Kobayashi et al.[35] These mice also exhibit hypergastrinemia and are insensitive to exogenous gastrin in acid secretion. In contrast to the HDC-deficient mice, $H_2$ receptor–deficient mice have been found to show marked hypertrophy of the gastric mucosa. Our hypotheses for the trophic effect of gastrin on the gastric mucosal cells are as follows. One possibility is that histamine may be a negative modulator of the proliferative effect of gastrin by suppressing hypertrophy via the $H_2$ receptors. Another possibility is that histamine may be essential for hypertrophy induced by hypergastrinemia and may act on histamine receptors other than the $H_2$ receptors. We propose that the latter is more likely because the HDC-deficient mice do not exhibit gastric hypertrophy despite their hypergastrinemia, and a higher amount of gastric histamine was detected in the $H_2$ receptor–deficient mice. A comparative study of the $H_2$- and HDC-deficient mice should provide valuable insights into the development of therapeutic strategies against gastric carcinoid formation.

The maximum levels of acid secretion in HDC-deficient mice were significantly higher than that of the wild-type mice. Maximum acid secretion is currently considered to reflect the capacity of translocation of the $H^+$, $K^+$-ATPase from the intracellular tubulovesicular compartment to the canaliculi of parietal cells.[36,37] Although the expression of the $H^+$, $K^+$-ATPase $\alpha$ subunit was not increased in the mutant mice, increased levels of the translocation may be achieved in the parietal cells of the mutant mice because the decreased histamine levels in the mutant mice may induce the translocation machinery or down-regulate the relocation machinery. Further investigations are required to clarify this last point.

Takeuchi et al. recently demonstrated that prolonged treatment of rabbits with an $H_2$ receptor antagonist induced a 2-fold increase in $H_2$ receptor density in parietal cells, without any change in affinity for [$^{125}$I]aminopotentidine.[24] We also confirmed an increase in the number of $H_2$ receptors with low binding affinity for a specific ligand, [$^3$H]tiotidine, in the gastric mucosal membrane fraction of the mutant mice. In addition, a slight increase in G$s\alpha$ expression was observed in the gastric membranes of the mutant mice. Our observation that there were no differences in serum histamine concentration after histamine injection in the wild-type and mutant mice excludes the possibility that the availability of histamine is altered in the mutants. It is likely that the signal transduction pathway via the $H_2$ receptors has been converted to a highly sensitive state in HDC-deficient mice. Analyses of certain phenotypes observed in HDC-deficient mice should contribute towards the development of therapies for rebound acid hypersecretion, which is observed after the abrupt withdrawal of prolonged $H_2$ receptor blockades by $H_2$ antagonists.

In summary, we have shown that enhanced acid secretion is induced by histamine in the HDC-deficient mice. This study also confirms the concept that histamine production is essential for the gastric acid secretion induced by gastrin, but not for that induced by carbachol. Our HDC-deficient mice should represent a suitable model for further functional analyses of the correlation between histamine and the other acid secretagogues.

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# Augmentation of Receptor-Mediated Adenylyl Cyclase Activity by Gi-Coupled Prostaglandin Receptor Subtype EP3 in a Gβγ Subunit-Independent Manner

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**We previously demonstrated that the mouse EP3β receptor and its C-terminal tail-truncated receptor (abbreviated T-335) expressed in Chinese hamster ovary cells showed agonist-dependent and fully constitutive Gi activity in forskolin-stimulated cAMP accumulation, respectively. Here we examined the effect of the EP3β receptor or T-335 receptor on adenylyl cyclase activity stimulated by the Gs-coupled EP2 subtype receptor in COS-7 cells. As a result, sulprostone, a selective EP3 agonist, dose dependently augmented butaprost-stimulated adenylyl cyclase activity in EP3β receptor- or T-335 receptor-expressing COS-7 cells. However, such adenylyl cyclase augmentation was not attenuated by either pertussis toxin treatment or expression of the PH domain of rat βARK1, which serves as a scavenger of Gβγ subunits, but was partially attenuated by treatment with either 1,2-bis(*o*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetra(acetoxymethyl)ester, an intracellular $Ca^{2+}$ chelator, or W-7, a calmodulin inhibitor. These findings suggest that the C-terminal tail of the EP3β receptor is not essentially involved in activation of EP2 receptor-stimulated adenylyl cyclase in a $Ca^{2+}$/calmodulin-dependent but Gβγ subunit-independent manner.** © 2002 Elsevier Science

*Key Words:* **$PGE_2$ receptor; EP3 subtype; EP2 subtype; adenylyl cyclase; Gi protein; Gβγ subunits; calmodulin; COS-7 cells.**

The interaction of cell surface hormone receptors with heterotrimeric G proteins is crucial for hormonal action (1). Most G protein coupled receptors (GPCRs) share conserved structural features, consisting of seven-transmembrane-spanning domains and three intracellular loops and one C-terminal tail (2). In a number of GPCRs, several regions in the cytoplasmic domains were reported to contribute directly or indirectly to G protein coupling (3). The specific regions in the second and third intracellular loops were reported to function in G protein coupling (4). However, the precise molecular mechanism for regulation of the receptor-induced G-protein activation is still poorly understood.

We previously cloned the mouse prostaglandin (PG) EP3 receptor and demonstrated that this receptor is a G protein coupled rhodopsin-type receptor that engages in inhibition of adenylyl cyclase (5). Furthermore, we identified the three isoforms of the EP3 receptor, EP3α, β, and γ, with different C-terminal tails, which were produced through alternative splicing (6, 7) and differed in agonist-independent constitutive Gi activity (8, 9). The EP3β receptor exhibits a full agonist-dependent Gi activity. In addition, the C-terminal tail–truncated receptor, T-335, showed only agonist-independent constitutive Gi activity. This suggests that the core of the EP3 receptor has an ability to associate with and activate Gi, and then the C-terminal tails of the EP3 receptor can suppress the activation of Gi (8).

Although Gi/o-coupled receptors generally act to inhibit adenylyl cyclase, it was also reported that long-term activation of the inhibitory receptors with agonists leads to an increase in adenylyl cyclase activity in a time- and dose-dependent manner (10, 11). This phenomenon is termed adenylyl cyclase superactivation or sensitization, which is mediated by adenylyl cyclase isozymes such as adenylyl cyclase II, directly being activated by Gβγ subunits derived from activation of Gi/o protein (12).

Abbreviations used: PG, prostaglandin; G protein, heterotrimeric GTP-binding protein; CHO, Chinese hamster ovary; COOH, carboxyl; PT, pertussis toxin; BAPTA/AM, 1,2-bis(o-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid tetra(acetoxymethyl)ester.

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To assess the role of the C-terminal tail of the EP3β receptor in regulation of receptor-medicated adenylyl cyclase activity, we examined the effect of EP3 receptor on adenylyl cyclase activity in EP2-receptor expressing COS-7 cells. As a result, the adenylyl cyclase activity of the EP2 receptor was augmented EP3 agonist-dependently in T-335 expressing cells, and augmentation was closely related to the activity of $Ca^{2+}$/calmodulin but not the Gi/o-derived Gβγ subunits.

## MATERIALS AND METHODS

*Materials.* The cDNAs for the mouse EP2 receptor (13) and EP3 receptor (5) were cloned in our laboratory. The truncated T-335 receptor was constructed as described previously (14). The cDNA for the rat LH/CG-R (15) was from Dr. D. L. Segaroff of the Department of Physiology and Biophysics, University of Iowa College of Medicine. LipofectAMINE PLUS was obtained from Life Technologies, Inc. M & B 28767, butaprost, and sulprostone were from Dr. M. P. L. Caton of Rhone-Poulenc Ltd. The $^{125}$I-labeled cAMP assay system was obtained from Amersham Corp. Pertussis toxin (PT) was obtained from Seikagaku Kogyo (Tokyo, Japan). Rabbit polyclonal anti-GRK2 (C-15) and rabbit polyclonal anti-A cyclase III (C-20) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA).

*DNA construction.* The peptide-encoding cDNA fragment for the PH domain of rat βARK1 ($Gly^{495}$-$Leu^{689}$) (16) were constructed by means of a polymerase chain reaction-mediated mutagenesis technique. The cDNAs encoding mouse EP2 receptor, rat LH/CG-receptor, EP3 receptor, T-335 receptor and the PH domain of rat βARK1 were subcloned into pcDNA3 (Invitrogen) eukaryotic expression vector.

*Cell culture and transient expression of receptors in COS-7 cells.* COS-7 cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% heat inactivated fetal bovine serum under humidified air containing 5% $CO_2$ at 37°C. For transfection using the LipofectAMINE PLUS reagent, cells in 60-mm tissue culture dishes were incubated at 37°C with a transfection mixture composed of 2.5 ml of serum-free DMEM containing 8 μg of DNA/dish, 10 μl of LipofectAMINE and 8 μl of PLUS reagent. After 3 h, the medium was changed to 3 ml of DMEM containing 10% heat inactivated fetal bovine serum. In cAMP assay COS-7 monolayers were trypsinized 24 h after transfection, and aliquots of recovered cells were transferred to 24-well tissue culture plates.

*Expression of the βARK1 PH domain and adenylyl cyclase III.* COS-7 cells were transiently cotransfected with increasing amounts of plasmid DNA encoding the PH domain of the rat βARK1. Expression of the βARK1 PH domain was determined by immunoblot analysis of whole cell detergent lysates using rabbit polyclonal anti-GRK2 (C-15). The PH domain expressing cells were harvested and washed twice in PBS. The cell pellet was suspended in 1 ml of RIPA buffer containing 30 mM Hepes–NaOH (pH 7.3), 150 mM NaCl, 1% Triton X-100, 1% deoxycholate and 0.1% SDS and incubated for 1 h at 4°C. For protection against proteolytic degradation, a mixture of protease inhibitors (0.2 mM PMSF, 100 μM benzamidine, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml E-64, and 1 μg/ml pepstatin A) was added. The mixture was then centrifuged at 100,000*g* for 10 min at 4°C. The resulting supernatant was dissolved in Laemmli buffer and heated for 5 min at 100°C. Aliquots (10 μg proteins) were then subjected to SDS–PAGE (15%) as described by Laemmli (17), and the separated proteins were transferred electrophoretically to a PVDF membrane in 25 mM Tris base containing 40 mM 6-aminohexanoic acid, 0.02% SDS and 20% methanol at room temperature for 30 min at 15 V. The membrane was rinsed in Tris-buffered saline (TBS) containing 20 mM Tris–HCl (pH 7.5) and 150 mM NaCl, and then preincubated overnight in TBS containing 5% non-fat milk at 4°C. The membrane was then incubated with anti-GRK2 (C-15) (1:200) antibody in TBS containing 5% nonfat milk for 1 h at 37°C. The membrane was washed three times with TTBS (TBS containing 0.05% Tween 20) at room temperature. The membrane was incubated with peroxidase-conjugated anti-rabbit IgG in TTBS for 1 h at room temperature, and then stained with the ECL Western blot detection reagent.

Expression of the adenylyl cyclase III was determined by immunoblot analysis of whole-cell detergent lysates using rabbit polyclonal anti-A cyclase III (C-20).

*Measurement of cAMP formation.* Cyclic AMP levels in COS-7 cells were determined as reported previously (18). The receptor-expressing COS-7 cells were pretreated with or without W-7 for 10 min in Hepes-buffered saline [140 mM NaCl, 4.7 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM $KH_2PO_4$, 11 mM glucose, and 15 mM Hepes (pH 7.4)], or BAPT/AM for 30 min in $Ca^{2+}$-free Hepes-buffered saline containing 5 mM EGTA in place of 2.2 mM $CaCl_2$. The cells were washed and preincubated for 10 min with 10 μM indomethacine in Hepes-buffered saline. Reactions were started by addition of test agents along with 100 μM Ro-20-1724. After incubation for 10 min at 37°C, reactions were terminated by the addition of 10% trichloroacetic acid. The content of cAMP was measured by radioimmunoassay with an Amersham cAMP assay system.

*ADP ribosylation of membrane with PT.* The preparation and ADP-ribosylation assay of crude membrane fractions were conducted by the method of Ui *et al.* (19). The COS-7 cells were treated with various doses of PT for 15 h. The treated cells were harvested and washed twice in PBS. The cell pellet was homogenized in 1 ml of Tris-buffered saline containing 20 mM Tris–HCl (pH 7.5), 1 mM EDTA, 10 mM $MgCl_2$, 1 mM DTT, 0.2 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 100 μM benzamidine using a Potter-type Teflon glass homogenizer, and then the mixture was centrifuged at 100,000 × g for 10 min at 4°C. The resulting pellets were used as the crude membrane fractions. The crude membrane fractions (20 μg of protein each) were incubated with 10 μg/ml of PT for 40 min at 30°C in 50 μl of 50 mM Tris–HCl (pH 7.5) containing 1 mM EDTA, 10 mM thymidine, 2.5 mM $MgCl_2$, 1 mM ADP-ribose, 15 mM isonicotinohydrazide, 2.5 μM [α-$^{32}$P]NAD (30 Ci/mmol), and 0.05% digitonin. The radiolabeled membranes were dissolved in Laemmli buffer and heated for 5 min at 100°C. Aliquots (6 μg each) were then subjected to SDS–PAGE (10%), as described by Laemmli (17). The gel was dried in a gel drier and exposed to X-ray film for 6 h at room temperature.

## RESULTS

### *Sulprostone-Induced Augmentation of Butaprost-Stimulated cAMP Formation in EP3β Receptor- or T-335 Receptor-Expressing COS-7 Cells*

In COS-7 cells expressing the EP3β receptor together with the EP2 receptor, $10^{-6}$ M butaprost, an EP2-specific agonist, elicited a significant cAMP accumulation. $10^{-7}$ M sulprostone, an EP3 agonist, further augmented butaprost-induced cAMP accumulation (Fig. 1A). We previously reported that a mutant of EP3 receptor with a deletion in the variable C-terminal tail, named T-335, exhibited constitutive Gi activity in CHO cells (8). However, in COS-7 cells expressing T-335 receptor together with EP2 receptor, butaprost increased cAMP accumulation, whose level was similar to that of cells expressing EP3β together with EP2 receptor. Sulprostone treatment resulted in enhancement of butaprost-induced cAMP accumulation (Fig.

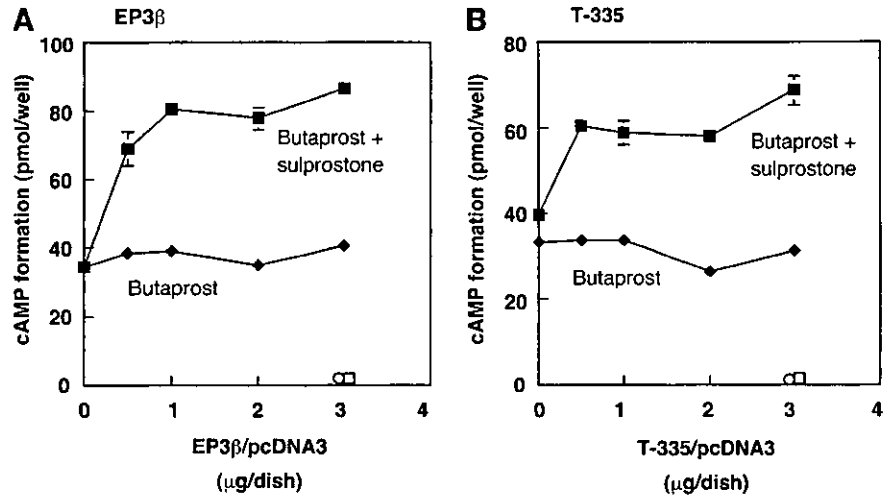


**FIG. 1.** Sulprostone-induced augmentation of butaprost-stimulated cAMP formation in EP3β receptor- or T-335 receptor-expressing COS-7 cells. COS-7 cells were transiently cotransfected with increasing amounts of plasmid DNA encoding EP3β receptor (A) or T-335 receptor (B) (0–3 μg/dish) with or without EP2/pcDNA3 (1 μg/dish). They were incubated at 37°C for 10 min with 1 μM butaprost in the absence (closed diamonds) or presence (closed squares) of 0.1 μM sulprostone. cAMP formations were determined as described under Materials and Methods. The control cells transfected the plasmid DNA encoding EP3 receptor (3 μg/dish) were incubated at 37°C for 10 min with 0.1 μM sulprostone in the absence (open circles) or presence (open squares) of 1 μM butaprost. Values are means ± SE of triplicate experiments.

1B). In the preliminary experiment, sulprostone dose-dependently enhanced the butaprost-stimulated cAMP accumulation in EP3β- or T-335- transfected COS-7 cells, but the half maximal concentration for EP3β mediated augmentation ($1 \times 10^{-8}$ M) were 2 orders of magnitude higher than for EP3β mediated Gi activity ($1 \times 10^{-10}$ M) (data not shown). Sulprostone alone did not stimulate adenylyl cyclase activity in COS-7 cells expressing the EP3β receptor or T-335 receptor alone (open circle in Fig. 1), suggesting that the adenylyl cyclase augmentation did not result from the direct activation of Gs protein via the EP3 receptor. Similar results were obtained when another EP3-agonist, M & B 28767, was used instead of sulprostone. Furthermore, the effects of EP3 agonists were obtained when LH/CG receptor-cDNA was used instead of the EP2-cDNA, followed by stimulation of LH (data not shown). These results suggested that stimulation of adenylyl cyclase by the EP3 receptor is irrespective of the Gs activation input signals.

*Effects of Pertussis Toxin and PH Domain of Rat βARK1 on Sulprostone-Induced Augmentation of Butaprost-Stimulated cAMP Formation in EP3β Receptor- or T-335 Receptor-Expressing COS-7 Cells*

Recently, it was reported that augmentation of Gs activity was achieved through Gβγ subunits resulting from the Gi/o protein activation in COS-7 cells (12). Therefore, we investigated whether the current findings included this machinery. Interestingly, it was found that PT failed to reverse the augmentation in adenylyl cyclase activity in COS-7 cells expressing the EP3β receptor or T-335 receptors (Fig. 2). These results suggest that EP3 receptor-involved adenylyl cyclase activation appears to be independent of Gi activation. Furthermore, when we transfected COS-7 cells with a minigene containing PH domain of rat βARK1 as a "decoy" of Gβγ dimmers in combination with EP3 and EP2 receptors, the treatment did not affect the levels of sulprostone-induced augmentation (Fig. 3). These findings suggested that the adenylyl cyclase augmentation was not due to Gβγ subunits resulting from the Gi activation by the EP3β receptor.

*Effect of BAPTA/AM, Intracellular $Ca^{2+}$ Chelator, and W-7, Calmodulin Inhibitor, on Sulprostone-Induced Augmentation of Butaprost-Stimulated cAMP Formation in EP3β Receptor- or T-335 Receptor-Expressing COS-7 Cells*

To clarify which signaling pathways are involved in EP3 receptor-elicited adenylyl cyclase superactivation, we examined the effects of intracellular $Ca^{2+}$ chelator, BAPTA/AM (20), and calmodulin inhibitor, W-7. As a result, it was found that sulprostone-induced augmentation was lost by treatment of each receptor-expressing COS-7 cells with BAPTA/AM, although this treatment slightly suppressed the basal activity by butaprost-induced adenylyl cyclase (Fig. 4). Recently, it was reported that the some isozymes of adenylyl cyclase, such as types I, III, and VIII, are stimulated by a high concentration of $Ca^{2+}$/calmodulin in the pres-

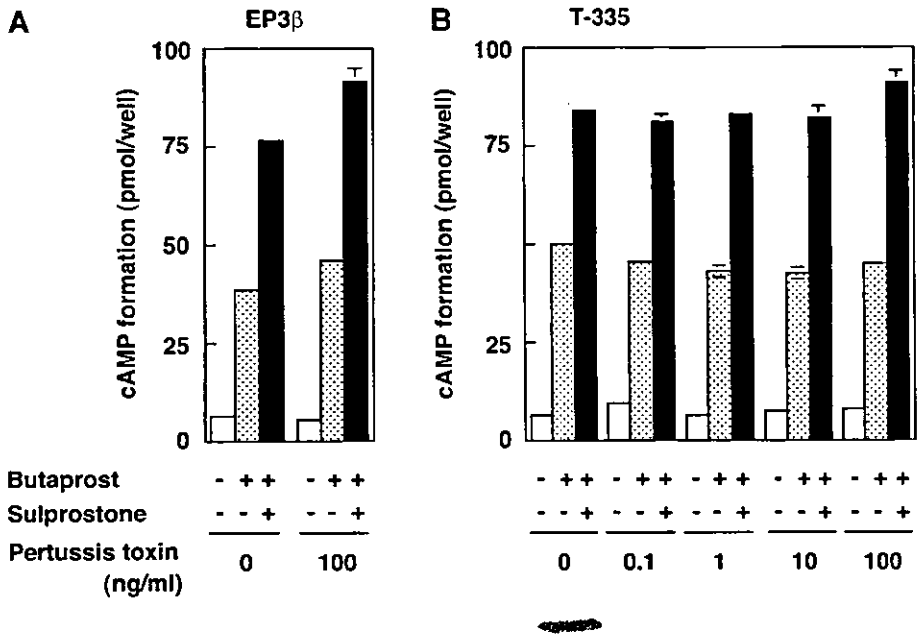


**FIG. 2.** Effect of pertussis toxin on sulprostone-induced augmentation of butaprost-stimulated cAMP formation in EP3β receptor- or T-335 receptor-expressing COS-7 cells. COS-7 cells were transiently cotransfected with plasmid DNA encoding EP3β receptor (A) or T-335 receptor (B) (1 μg/dish) with EP2/pcDNA3 (1 μg/dish). COS-7 cells expressing each receptor were pretreated with the indicated doses of PT (0–100 ng/ml) for 15 h. Then the treated cells were incubated at 37°C for 10 min with 1 μM butaprost in the absence or presence of 1 μM sulprostone. cAMP formations were determined as described under Materials and Methods. Values are shown as means ± SE of triplicate experiments. The lower panel shows the ADP-ribosylation of Gi protein. The crude membrane fractions from the cells pretreated with the indicated doses of PT as described above were incubated with [α-$^{32}$P]NAD and PT, and then subjected to SDS–PAGE. The band of the 41-kDa protein on an autoradiogram is shown. Autoradiogram was obtained after 6 h exposure to X-ray film.

ence of Gαs (21, 22). When we treated the each receptor-expressing COS-7 cells with W-7, the treatment attenuated the sulprostone-induced augmentation of butaprost-stimulated cAMP formation (Fig. 5). Furthermore, since W-7 itself had no effects on basal activity of butaprost-induced adenylyl cyclase, stimulation of adenylyl cyclase is unlikely to result from activation of $Ca^{2+}$/calmodulin-sensitive adenylyl cyclase.

## DISCUSSION

One of the most significant findings in the present study was that the mouse EP3 receptor could be exclusively coupled to augmentation of receptor-mediated adenylyl cyclase activity, when expressed in COS-7 cells. Augmentation of adenylyl cyclase in COS-7 cells was previously reported in various Gi-coupled receptors such as α2 adrenoceptor, and its mechanism was suspected to be an increase of adenylyl cyclase type II activity by direct interaction of Gβγ subunits resulting from activation of Gi/o proteins (12). Such phenomena are termed adenylyl cyclase superactivation. However, the current superactivation of adenylyl cyclase was insensitive to PT treatment and the PH domain overexpression (Figs. 2 and 3), indicating that adenylyl

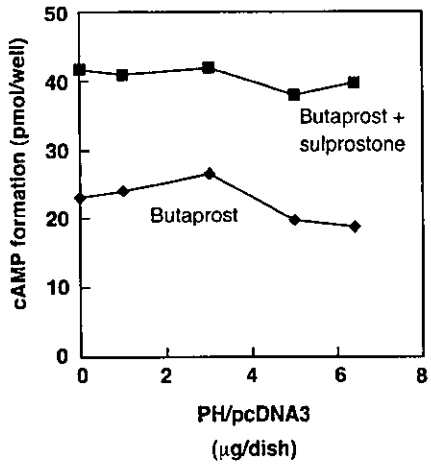


**FIG. 3.** Effect of the PH domain of rat βARK1 on sulprostone-induced augmentation of butaprost-stimulated cAMP formation in T-335 receptor-expressing COS-7 cells. COS-7 cells were transiently cotransfected with increasing amounts of plasmid DNA encoding the PH domain of rat βARK1 (0–6.4 μg/dish) with EP2/pcDNA3 (0.8 μg/dish) and T-335/pcDNA3 (0.8 μg/dish). They were incubated at 37°C for 10 min with 1 μM butaprost in the absence (closed diamonds) or presence (closed squares) of 1 μM sulprostone, and cAMP formations were determined as described under Materials and Methods. Values are means ± SE of triplicate experiments.

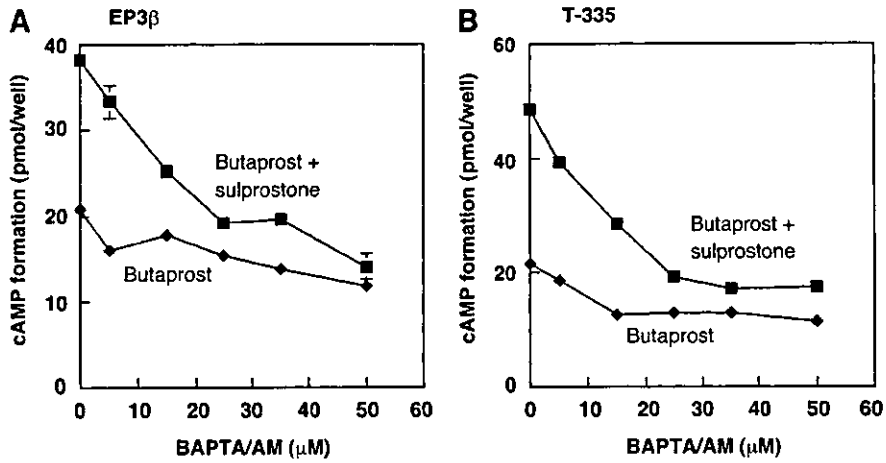


**FIG. 4.** Effect of BAPTA/AM on sulprostone-induced augmentation of butaprost-stimulated cAMP formation in EP3β receptor- or T-335 receptor-expressing COS-7 cells. COS-7 cells were transiently cotransfected with plasmid DNA encoding EP3β receptor (A) or T-335 receptor (B) (1 μg/dish) with EP2/pcDNA3 (1 μg/dish). After cells were pretreated with the indicated concentrations of BAPTA/AM in $Ca^{2+}$-free buffered saline for 30 min, they were incubated with 1 μM butaprost with (closed squares) or without (closed diamonds) 1 μM sulprostone. cAMP formations were determined as described under Materials and Methods. Values are means ± SE of triplicate experiments.

cyclase superactivation by the EP3β receptor appears to be mediated via a novel signal pathway without the involvement of Gβγ subunits. The inhibitory effects of BAPTA/AM and W-7 support the hypothesis that adenylyl cyclase superactivation via the EP3 receptor should be achieved via a signaling pathway relating to the $Ca^{2+}$/calmodulin-involved reactions (Figs. 4 and 5). We previously reported that the activation of mouse EP3α and EP3β receptors leads to $Ca^{2+}$ mobilization in a PT-sensitive manner in COS-7 cells (23). Since the current adenylyl cyclase superactivation by the EP3β receptor was conducted $Ca^{2+}$/calmodulin pathway in a PT-insensitive manner, indicating that EP3β receptor could be linked to $Ca^{2+}$ mobilization via activation of any PT-insensitive G proteins. Recently, nine distinct isozymes of adenylyl cyclase have been cloned. These isozymes differ in their properties, including their capacity to be inhibited or stimulated by G protein αi, αs, and βγ subunits, as well as by protein kinase C, and $Ca^{2+}$/calmodulin (20, 21). Furthermore, the adenylyl cyclase III has been reported to be stimulated by a high concentration of $Ca^{2+}$/calmodulin in the presence of Gαs, and to be unaffected by Gβγ subunits (24). Indeed the adenylyl cyclase III was expressed in COS-7 cells (data not shown), then it can be that the activation of EP3β receptor leads to superactivation of this cyclase.

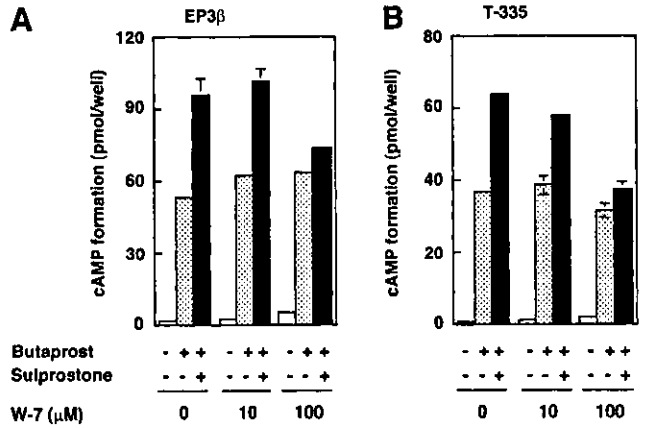


**FIG. 5.** Effect of W-7 on sulprostone-induced augmentation of butaprost-stimulated cAMP formation in EP3β receptor- or T-335 receptor-expressing COS-7 cells. COS-7 cells were transiently cotransfected with plasmid DNA encoding EP3β receptor (A) or T-335 receptor (B) (1 μg/dish) with EP2/pcDNA3 (1 μg/dish). After cells were pretreated with the indicated concentrations of W-7 for 10 min, they were incubated with 1 μM butaprost with or without 1 μM sulprostone. cAMP formations were determined as described under Materials and Methods. Values are means ± SE of triplicate experiments.

We previously demonstrated that EP3β receptor is entirely coupled to Gi activation resulting in inhibition of forskolin-stimulated adenylyl cyclase in CHO cells (6). Furthermore, we showed that removal of the C-terminal tail of EP3β receptor resulted in agonist-independent constitutive Gi activity in CHO cells (8). Indeed, similar results were obtained when we transiently introduced EP3β receptor cDNA or T-335 receptor cDNA together with EP2 receptor cDNA into HEK293 cells; EP3β and T-335 receptors showed Gi activity against butaprost-stimulated adenylyl cyclase in agonist-dependent and agonist-independent manners, respectively, and these activities were PT-sensitive (data not shown). From these results together with the previous our findings that EP3α and EP3γ receptors have different constitutive activity, it is suggested that the "common" structure of EP3 receptor contains domains necessary for the association and activation of Gi proteins, and the C-terminal tails have

a role for inhibition of leakage of constitutive activity. However, in the present study, EP3β and T-335 receptors raised similar profiles in the expression of adenylyl cyclase superactivation in COS-7 cells. Subsequently, the common structure in EP3 receptors has potential sites for eliciting two signaling pathways in an agonist dependent manner; one is Gi activity and the other is adenylyl cyclase superactivation, the latter effect is unlikely to be elicited by the direct involvement of Gs protein and adenylyl cyclase. Since C-terminal tails of EP3 receptors are dispensable in the latter signaling pathway, the conformational feature of the common structure of EP3β receptor may be quite different at the expression of adenylyl cyclase augmentation from the Gi activation.

Recently, Audoly *et al.* reported that the rabbit EP3 receptors can couple to activation of cAMP response element (CRE)-mediated gene transcription, which is PT-insensitive in HEK 293 cells (25). They also showed that the C-terminus-truncated isoform of the rabbit EP3 receptor can elicit this activation in an agonist-dependent manner although their $EC_{50}$ values are 15-fold higher than that in Gi activity. Furthermore, based on the increase in intracellular $Ca^{2+}$, they suspected that CRE activation is mediated, in part, by a $Ca^{2+}$-dependent kinase pathway. It is currently unknown whether the signaling pathway they found is associated with the adenylyl cyclase superactivation in COS-7 cells, although they failed to detect cAMP accumulation by sulprostone alone in HEK 293 cells. Thus, the signal transduction properties of the EP3 receptor appear to be dependent on the cellular background in which they are expressed. However, it remains to be clarified which elements of cellular background determine the signaling pathway of EP3 receptors.

In summary, the C-terminal tail of the EP3β receptor is not essentially involved in activation of EP2 receptor-stimulated adenylyl cyclase in a $Ca^{2+}$/calmodulin-dependent manner, while these C-terminal regions of EP3 receptors play an important role in the inhibition of the activation of Gi. This study will contribute not only to the understanding of the heterogeneity of $PGE_2$ actions but also to elucidate the molecular mechanism of G protein activation induced by EP3β receptor.

## ACKNOWLEDGMENTS

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# Plasma extravasation induced by dietary supplemented histamine in histamine-free mice

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Histidine decarboxylase (HDC) synthesizes endogenous histamine from histidine in mammals. To evaluate the role of histamine in skin allergic reaction, we used HDC gene knockout mice lacking histamine. No plasma extravasation reaction was observed in HDC(–/–) mice after passive cutaneous anaphylaxis (PCA) test. Compound 48/80, a mast cell granule depletor, produced plasma extravasation in HDC(+/+) mice but no extravasation in HDC(–/–) mice. Interestingly, orally administered histamine was distributed in the skin in HDC(–/–) mice and in these histamine-supplemented mice the plasma extravasation reaction was observed after the injection of compound 48/80 and the PCA test. Cultured bone marrow-derived mast cells of HDC(–/–) mice took up histamine from the histamine-supplemented medium into the secretory granules. The absorbed histamine was released in response to the same antigen and antibody combination used as in PCA test. In contrast to the immediate-type response, the delayed-type hypersensitive response, observed as a thickening of the ear skin after trinitrochlorobenzene challenge (following sensitization), showed no differences between HDC(+/+) and HDC(–/–) mice. Therefore, among the allergic skin reactions, histamine is revealed to be an important mediator especially for the plasma extravasation in an immediate-type allergy model.

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**Key words:** Mast cell / Mouse / Knockout / Hypersensitivity / Edema

## 1 Introduction

Histamine is synthesized by a unique enzymatic reaction with L-histidine decarboxylase (HDC). In pathological skin conditions, histamine is involved in the induction of itching, flaring and edema [1, 2]. However, there has been no consensus about the mediator directly responsible for the extravasation reaction. In addition, various studies have suggested a close relationship between histamine production and wound healing [3], embryogenesis [4], hematopoiesis [5] and malignant growth [6, 7]. Accordingly, we established knockout mice with defective histamine synthesis by introducing a mutation into the *HDC* gene that resulted in mice without HDC activity. The decarboxylase activity of the enzyme depends on the presence of a co-enzyme, pyridoxal 5'-phosphate, which binds to HDC at a putative binding site (TFNPSKW) [8]. Based on this information, we constructed a targeting vector that replaces this binding site with a neomycin-resistance cassette.

The biological role of histamine has been extensively studied using pharmacological approaches with specific receptor agonists and antagonists [9] or with histamine synthesis inhibitors [10]. Due to the overlapping and sometimes antagonistic function of the receptors in the

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**Abbreviations: HDC:** Histidine decarboxylase **TNCB:** Trinitrochlorobenzene **FMH:** Fluoromethyl histidine **CH:** Contact hypersensitivity **BMMC:** Bone marrow-derived mast cell **LDH:** Lactate dehydrogenase **VMAT:** Vesicular monoamine transporter **bHEX:** β-Hexosaminidase **L diet:** Low-histamine diet **L+HA:** Histamine-enriched L diet **PCA:** Passive cutaneous anaphylaxis

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presence of endogenous histamine, receptor-blocking alone cannot achieve complete elimination of the histamine system. In addition, histamine receptors might cryptically bind to substances other than histamine. Although the histamine synthesis blocker α-fluoromethyl histidine (α-FMH) significantly decreases the level of histamine in various organs, it is difficult to achieve complete and long-lasting elimination of histamine *in vivo* in this manner. Therefore, we concentrated on the elimination of histamine biosynthesis by an ES cell-mediated gene targeting approach.

We have already described gene-targeted mice lacking HDC activity in the assessment of histamine activity *in vivo* [11]. To assess the role of histamine in models for skin allergy, we chose two kinds of models that are frequently used as models for human skin allergic disease: one is the immediate-type skin reaction via IgE antibody (Type I allergic reaction), the other is the contact hypersensitivity (CH) reaction as a form of delayed-type hypersensitivity (DTH; Type IV allergic reaction). These are commonly used disease models for urticaria and contact dermatitis, respectively [1]. We demonstrate that only the immediate-type skin reaction was strongly affected by histamine and that the CH appeared to be weakly affected by histamine. Also we show that histamine could be absorbed from the digestive tract and presumably distributed into the mast cell granules. These mice provide an excellent *in vivo* system for gaining insight into the essential roles of histamine in a broad range of physiological and pathological processes with an *in vivo* rescue using dietary supplemented histamine.

## 2 Results

### 2.1 Absorption of dietary supplemented histamine by HDC(–/–) mice

To assess absorption of dietary supplemented histamine in HDC-deficient animals, histamine levels and the histamine synthesis activity were measured in various organs of HDC(–/–) mice under two different dietary conditions: a low-histamine diet (L) and a histamine-enriched diet (L+HA) (Table 1). The L+HA diet was prepared by adding histamine at 80 μmol per 1 g of L diet. The tissues of the HDC(–/–) animals lacked histamine synthesis activity. The histamine levels in various organs were practically zero except for that of brain. However, significantly high levels of histamine were found in every organ when the animals were kept on the L+HA diet for 7 days.

**Table 1.** Histamine content (H), HDC activity (HDC) and their dependence on different dietary conditions in organs of HDC(+/+) and HDC (–/–) mice[a]

| | | L diet (0.6 nmol histamine/g food) | | L + HA diet (80 μmol histamine/g food) |
|---|---|---|---|---|
| | | +/+ | –/– | –/– |
| Brain | H | 58.67±9.83 | 18.41±2.74 | 83.28±32.71 |
| | HDC | 0.24±0.04 | 0.00±0.00 | 0.00± 0.00 |
| Stomach | H | 4.36±0.50 | 0.10±0.05 | 4.92± 0.90 |
| | HDC | 1.98±0.22 | 0.03±0.02 | 0.06± 0.02 |
| Spleen | H | 0.94±0.52 | 0.04±0.01 | 6.78± 0.97 |
| | HDC | 0.01±0.00 | 0.01±0.00 | 0.01± 0.00 |
| Kidney | H | 2.11±1.28 | 0.01±0.00 | 3.40± 0.09 |
| | HDC | 18.03±0.01 | 0.01±0.00 | 0.02± 0.01 |
| Skin | H | 21.22±4.25 | 0.26±0.06 | 10.53± 0.65 |
| | HDC | 2.12±0.56 | 0.00±0.05 | 0.00± 0.00 |
| Plasma | H | 0.32±0.06 | 0.04±0.01 | 11.31± 1.25 |

a) Histamine levels are given in: pmol/g tissue for brain, nmol/g tissue for stomach, spleen, kidney and skin, and nmol/ml for plasma. HDC concentrations are given in pmol/ml protein/min, (mean ± SE, $n = 4$).