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ORIGINAL PAPER

Gastric acid secretion in cholecystokinin-1 receptor, -2 receptor, and -1, -2 receptor gene knockout mice

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Abstract Gastrin is important for stimulating acid secretion as well as differentiating gastric mucosal cells via cholecystokinin-2 receptors (CCK-2Rs). In turn, CCK acts preferably via CCK-1R to release somatostatin, and somatostatin has been postulated to exhibit a tonic inhibition of gastrin bioactivity. The present study was designed to examine the hypothesis that CCK-1R and 2R may act in opposite directions in gastric acid secretion. Having generated CCK-1R(−/−), 2R(−/−), and 1R(−/−)2R(−/−) mice, we examined the regulation of gastric acid secretion in four genotypes including wild-type mice. Parietal cells possess histamine receptors, muscarinic receptors, and CCK-2Rs. Since histamine increases cAMP and carbachol increases calcium, the responses of gastric acid secretion to graded doses of histamine, carbachol, and a combination of histamine + carbachol were determined. The sensitivity to histamine did not differ among the four genotypes, while the maximal acid secretion was lower in CCK-2R(−/−) mice than in wild-type mice. In addition, sensitivity to carbachol was impaired in mice without CCK-2R. The interaction of histamine and carbachol was conserved in all genotypes. In conclusion, CCK-2R is necessary to respond to carbachol as well as to produce the maximal acid secretion, while the role of CCK-1R in acid secretion is less important.

Keywords Acetylcholine · CCK · Knockout · Mice

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Introduction

Gastric acid secretion is regulated in a complex manner by neurocrine, endocrine, and paracrine signals. The primary source of gastric acid is the parietal cells, and the major peripheral stimuli acting on the parietal cells directly are histamine, acetylcholine, and gastrin [1]. Gastrin has been reported to stimulate acid secretion in two ways via gastrin/cholecystokinin-2 receptors (CCK-2Rs): (1) by acting on the enterochromaffin-like (ECL) cells to stimulate histamine release and de novo formation of histamine, and (2) by acting directly on the parietal cells to stimulate acid secretion [2, 3], although this latter effect is still controversial. The parietal cells possess three different types of receptors: CCK-2R, muscarinic 3 receptor (M3-R), and histamine 2 receptor (H2-R). Two cellular pathways of acid secretion in the parietal cells have been elucidated: increases in cAMP content and increases in calcium concentration. Histamine activates H2-Rs and increases cAMP in the parietal cells, resulting in gastric acid secretion. Gastrin and acetylcholine released from parasympathetic nerve terminals activate CCK-2R and M3-R, respectively, increase calcium concentrations in the parietal cells, and stimulate acid secretion [1, 2].

However, in a recent report [4] using L-histidine decarboxylase (HDC)-deficient (−/−) mice, which could not

synthesize histamine in the ECL cells, gastrin failed to stimulate acid secretion. Moreover, neither histamine nor gastrin stimulated gastric acid secretion in H2-R (−/−) mice [5]. This evidence indicates that histamine is a final mediator of acid secretion in mice when gastrin is administered. On the other hand, gastrin is known to be more important for the differentiation of gastric mucosal cells than for the acid secretion in mice [6, 7]. CCK-2R (−/−) mice showed a decrease in the numbers of parietal cells and ECL cells, and sustained hypergastrinemia [8, 9]. The numbers in gastrin-producing G cells were increased and the decrease in antral D cell numbers was observed as possibly due to the long-term elevation of gastric pH [8]. More recently, Chen et al. [10] reported that differentiation of gastric ECL cells is altered in CCK-2R (−/−) mice: ECL cells were replaced by an "ECL-like" cell type, characterized by a lack of secretory vesicles, and the histamine content and HDC activity in the oxyntic mucosa were low.

In turn, somatostatin was postulated to exhibit a tonic inhibition of parietal cells, ECL cells, and G cells in dogs. Canine somatostatin-producing D-cells possess CCK-1R [11], and CCK acts preferably via CCK-1R to release somatostatin [12]. We reported overexpression of CCK-2R in CCK-1R deficient (OLETF) rats [13]. Tachibana et al. [14] reported in the OLETF rats that CCK produced higher gastric acid secretion compared with control rats. These observations suggest that CCK-1R and 2R may act in opposite directions in gastric acid secretion. Two types of CCK receptors (CCKAR and CCK-BR) have been cloned [15], and recently renamed CCK-1R and CCK-2R [16]. CCK-1R binds sulfated CCK with 500- to 1,000-fold higher affinity than gastrin and non-sulfated CCK, while CCK-2R interacts with gastrin and CCK with almost the same affinity. Recently, we generated CCK-1R(−/−), 2R(−/−), and 1R(−/−)2R(−/−) mice [17–19]. Here, the role of CCK-1R and 2R in gastric acid secretion was determined using these three genotypes of mice as well as wild-type mice.

Materials and methods

The present experimental protocol was reviewed and approved by the appropriate committee of the Tokyo Metropolitan Institute of Gerontology. We also followed the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

Animals

The progenitor strain for CCK-1R(−/−) and 2R(−/−) mice was C57BL/6J. Backcrossing was carried out for more than seven generations. Three male CCK-1R(−/−) mice were bred with 12 female CCK-2R(−/−) mice to yield F1 progeny with the genotype CCK-1R(+/−)2R (+/−). Male and female F1 mice were then bred to yield progeny with nine genotypes: CCK-1R(+/+)2R(+/+), CCK-1R(+/+)2R(+/−), CCK-1R(+/+)2R(−/−), CCK-1R(+/−)2R(+/+), CCK-1R(+/−)2R(+/−), CCK-1R(+/−)2R(−/−), CCK-1R(−/−)2R(+/+), CCK-1R(−/−)2R(+/−) and CCK-1R(−/−)2R(−/−). Finally, male CCK-1R(−/−)2R(−/−) mice were then bred with female CCK-1R(−/−)2R(−/−) mice to obtain double knockout mice. CCK-1R(−/−) and CCK-2R(−/−) mice were selected from the above lines, and wild-type mice CCK-1R(+/+)2R(+/+) were selected at random from both the CCK-1R and CCK-2R lines [17–19]. Female mice at 6–8 months of age were used for the present experiments. The animals were maintained in individual cages in an air-conditioned room at 21°C, with 55 +/− 5% humidity, and with a 12 h light/dark photocycle (8:00AM–20:00PM) at Tokyo Metropolitan Institute of Gerontology. The cage size was 18 × 30 × 11 cm.

Materials and chemicals

Xylazine and ketamine were purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan), histamine and carbamylcholine (carbachol) from Sigma Chemical Co. (St Louis, MO, USA), and somatostatin-14 from Peptide Institute, Inc. (Osaka, Japan).

Experimental protocols

The mice fasted overnight, but had free access to water. After anesthetization by a mixture of xylazine and ketamine (11.3 and 8.7 mg/kg in, respectively), the trachea was cannulated. An overhead lamp was used to maintain core body temperature at 36 ~ 38°C. The abdomen was opened and the esophagus and pylorus were ligated. PE-50 tubing 0.58 mm inner diameter (ID), 0.965 mm outer diameter (OD) was inserted through an incision in the forestomach to infuse isotonic saline. Vinyl tubing (2.0 mm ID, 2.8 mm OD) was inserted in the antrum to collect stomach secretions [4]. After washing with 6–8 ml isotonic saline (37°C), an isotonic saline infusion was started at a rate of 0.2 ml/min.

In some animals, the right femoral vein was cannulated to infuse somatostatin-14.

Responses to graded doses of carbachol

Samples were collected every 10 min, and acid output (μmol H^+) was determined by titration using an autotitrator (TOA Electronics Ltd., Tokyo, Japan). After 60 min basal collection, carbachol (10, 50, and 100 μg/kg) was injected

subcutaneously in wild-type, CCK-1R(−/−), 2R(−/−), and 1R(−/−)2R(−/−) mice. Samples were collected subsequently for an additional 60 min.

We found slight differences in acid secretion in response to carbachol between CCK-2R(−/−) and CCK-1R(−/−)2R(−/−) mice. No dose of carbachol produced a significant increase in acid secretion in CCK-2R(−/−) mice; however, CCK-1R(−/−)2R(−/−) mice responded to 50 and 100 μg/kg carbachol, producing significant increases. Based upon a previous report [11] that CCK-1Rs are located in somatostatin-secreting cells of the stomach, we examined whether somatostatin could inhibit the substantial increase observed in CCK-1R(−/−)2R(−/−) mice. Hence, in CCK-1R(−/−)2R(−/−) mice, intravenous infusion of somatostain-14 (10 μg/kg per h) [20] was started 30 min before 50 μg/kg carbachol injection and continuously infused throughout the experimental period.

Responses to graded doses of histamine

After 60 min basal collection, histamine (0.1, 0.5 and 1.0 μg/kg was injected subcutaneously, and samples were collected as described above. The effect of a further high dose 10 mg/kg histamine was examined in wild-type and CCK-2R(−/−) mice only, because CCK-1R(−/−) and CCK-1R(−/−)2R(−/−) mice did not reach a sufficient age and were not available.

Responses to both histamine + carbachol

The pathways of histamine and carbachol in the parietal cells are different; histamine increases cAMP and carbachol increases calcium concentrations. As the interaction of cAMP and calcium is well known [21], the effects of carbachol (10 μg/kg) combined with histamine (0.1 or 1.0 μg/kg) were examined.

Statistical analysis

Values are expressed as means ± SE. The results were analyzed by multiple analysis of variance (MANOVA) with repeated measures to determine changes in gastric acid secretion with time, or by one-way or two-way ANOVA with respect to genotype and/or treatment, followed by Fisher's protected least significant differences tests. All P values computed were two-tailed, and P values <0.05 were regarded as statistically significant.

Results

The mean body weights were as follows: 24.37 ± 0.28 g (mean ± SE) for wild-type, 24.24 ± 0.49 g for CCK-1R(−/−), 24.28 ± 0.34 g for CCK-2R(−/−), and 23.23 ± 0.35 g for CCK-1R(−/−)2R(−/−) mice. There were no significant differences among genotypes.

Basal gastric acid secretion among four genotypes

The basal secretion level was estimated using the mean value during the 30 min before injection obtained from all experiments. When analyzed by one-way ANOVA, the basal gastric acid secretion was significantly different among the four genotypes [$F(3,241) = 7.071$, $P = 0.0001$] (Table 1). The value of CCK-2R(−/−) mice was significantly lower than those of the other three genotypes, and the value of CCK-1R(−/−)2R(−/−) mice was significantly lower than that of CCK-1R(−/−) mice by the multiple comparison test.

Effects of various doses of carbachol among four genotypes

The results of respective genotypes are shown in Fig. 1. When analyzed by MANOVA with repeated measures in each genotype, gastric acid secretion was significantly changed with respect to time in all genotypes [$F(2, 6) = 19.49$ for wild-type, 30.35 for CCK-1R(−/−), 6.78 for CCK-2R(−/−), and 10.73 for CCK-1R(−/−)2R(−/−) mice, $P < 0.0001$ for all F values]. However, since the stimulatory effect of carbachol showed a bell-shaped pattern, the F value with respect to dose was not significant in wild-type [$F(2,23) = 1.00$, $P = 0.38$], CCK-1R(−/−) [$F(2,14) = 3.39$, $P = 0.063$], and CCK-2R(−/−) mice [$F(2,22) = 1.31$, $P = 0.29$], but was significant in CCK-1R(−/−)2R(−/−) mice [$F(2,18) = 9.34$, $P = 0.0017$].

By the multiple comparison test, 50 μg/kg carbachol produced the highest increase while 10 and 100 μg/kg produced smaller increases (Fig. 1a, b, d). All three doses of carbachol significantly increased acid secretion in wild-type and CCK-1R(−/−) mice (Fig. 1a, b). By contrast, no

Table 1 Basal gastric acid secretion (μmol/10 min per 10 g body weight)

Genotype	Acid output
Wild-type (73)	0.41 ± 0.01
CCK-1R(−/−) (49)	0.45 ± 0.02
CCK-2R(−/−) (59)	0.30 ± 0.01*
CCK-1R(−/−)2R(−/−) (64)	0.38 ± 0.02**

Values are means ± SE; numbers in parentheses are the number of animals

$F(3,241) = 7.07$, $P = 0.0001$ by ANOVA; * significantly lower than other values, ** significantly lower than the value of CCK-1R(−/−) mice by the multiple comparison test

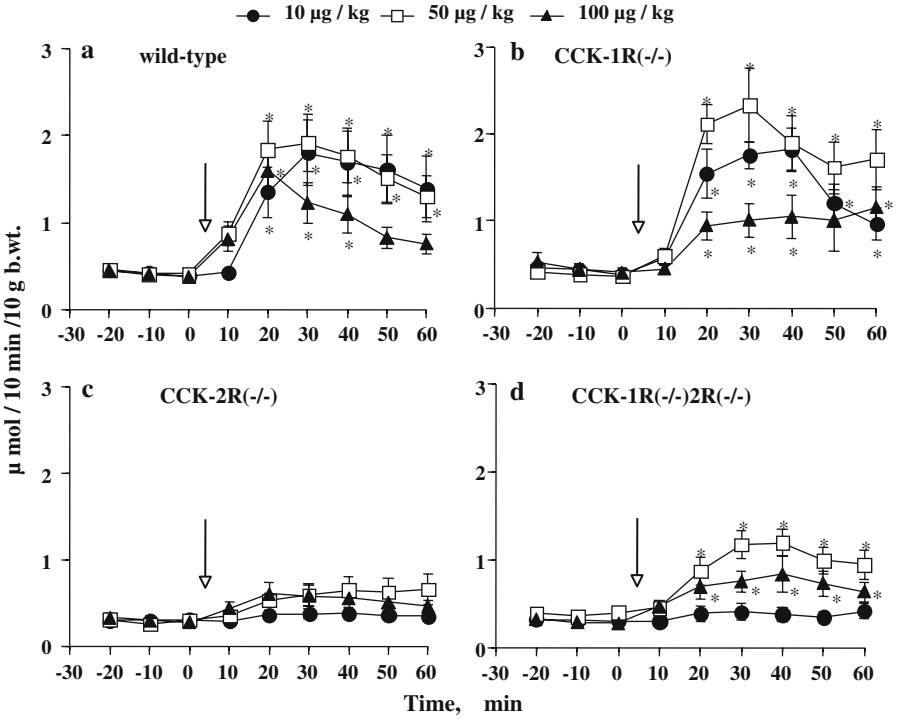

Fig. 1 Changes in gastric acid secretion in response to graded doses of carbachol (10, 50, and 100 μg/kg) in wild-type (**a**), CCK-1R(−/−) (**b**), CCK-2R(−/−) (**c**), and CCK-1R(−/−)2R(−/−) mice (**d**). The results of statistical analyses with respect to time are shown in the text. *Asterisks* indicate a significant difference from the respective value before carbachol injection by the multiple comparison test. The numbers of animals were 5–9 for each treatment. The *arrows* indicate the carbachol injection

dose of carbachol produced a significant increase in gastric acid secretion in CCK-2R(−/−) mice (Fig. 1c). Although 10 μg/kg carbachol injection did not produce a significant increase in CCK-1R(−/−)2R(−/−) mice, both 50 and 100 μg/kg carbachol significantly increased gastric acid secretion (Fig. 2d).

Intravenous administration of somatostatin-14 completely abolished carbachol (50 μg/kg)-stimulated acid secretion in CCK-1R(−/−)2R(−/−) mice (Fig. 2).

Effects of various doses of histamine among four genotypes

The lowest dose (0.1 μg/kg) did not affect acid secretion significantly in any genotype, but higher doses (0.5 and 1.0 μg/kg) stimulated gastric acid secretion in all genotypes. When analyzed by MANOVA with repeated measures, the responses to 1.0 μg/kg were significantly different with respect to time [$F(3,6) = 16.06$, $P < 0.0001$], but not to genotype [$F(3,27) = 0.488$, $P = 0.694$]. To compare the genotype differences more clearly, the increment above the basal level during the 60 min period after injection was calculated as follows: the sum of acid output during the 60 min − (acid output during 10 min before histamine injection × 6). The changes in incremental gastric acid secretion were significantly different with respect to dose [$F(3,91) = 43.55$, $P = 0.0001$], but not with respect to genotype [$F(3,91) = 0.34$, $P = 0.796$] (Fig. 3a).

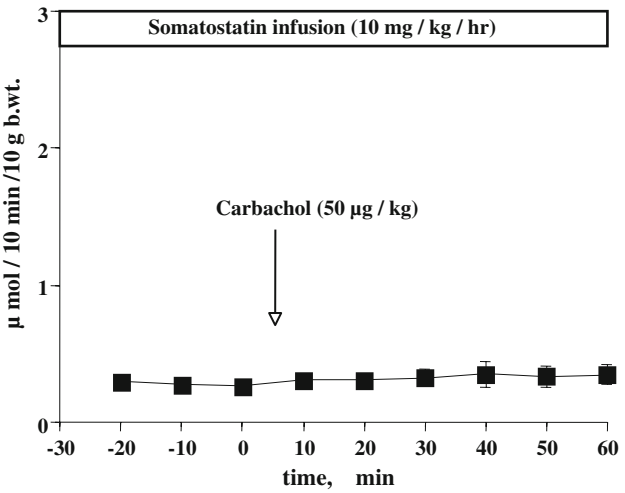

Fig. 2 The effect of 10 mg/kg per h of somatostatin-14 on gastric acid secretion produced by 50 μg/kg carbachol in CCK-1R(−/−)2R(−/−) mice. The effect of carbachol was completely abolished. $N = 6$. The *arrow* indicate the carbachol injection

Ten mg/kg histamine further increased gastric acid secretion in wild-type and CCK-2R(−/−) mice (Fig. 3b). The maximal value in wild-type mice was observed during the period 50–60 min post-injection (2.41 ± 0.26μmol/10 min/10 g body weight), and reached a value similar to that produced by 50 μg/kg carbachol as shown above. This highest dose of histamine also further increased gastric acid secretion in CCK-2R(−/−) mice, but the secretion was significantly lower than that in wild-type mice (Fig. 3b).

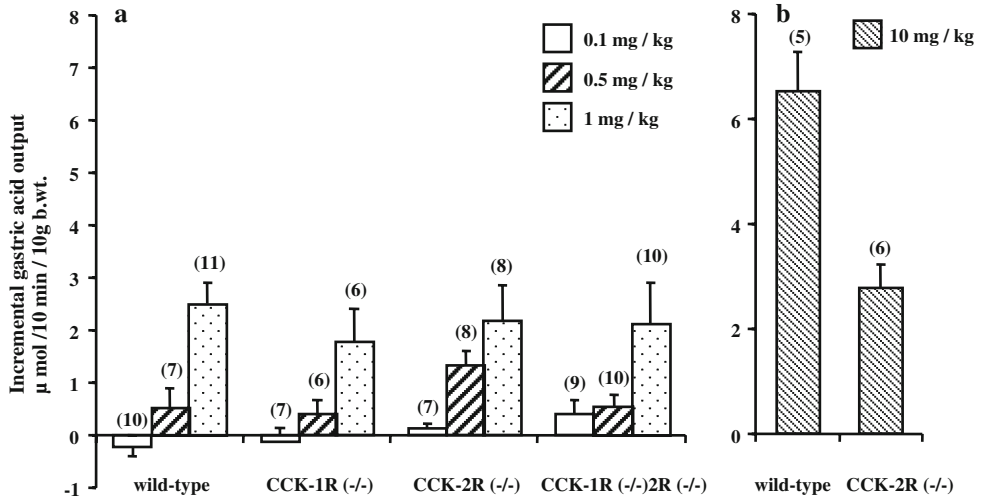

Fig. 3 Incremental gastric acid secretion stimulated by histamine. **a** Incremental gastric acid outputs during the 60 min period following injection by graded doses of histamine (0.1, 0.5, and 1.0 mg/kg) in four genotypes of mice. There were no significant differences among genotypes when analyzed by 2-way ANOVA. **b** Incremental acid outputs stimulated by 10 mg/kg of histamine in wild-type and CCK-2R(−/−) mice. The estimation methods and the results of statistical analysis are shown in the text. The numbers in parentheses are the number of animals

Effects of carbachol + histamine

In the case of administration of histamine 0.1 μg/kg + carbachol 10 μg/kg, the increments produced by histamine + carbachol were similar to those produced by 10 μg/kg of carbachol alone (data not shown). As shown in Fig. 3a, 0.1 mg/kg histamine alone produced no significant increase in any genotype.

However, the combination of histamine 1.0 mg/kg + carbachol 10 μg/kg further increased acid secretion in wild-type and CCK-1R(−/−) mice compared with values produced by either carbachol or histamine. Incremental outputs are shown in Table 2. Although 10 μg/kg carbachol did not produce a significant increase in CCK-2R(−/−) and CCK-1R(−/−)2R(−/−) mice, the mean value produced by histamine + carbachol was significantly higher than the value produced by histamine alone in CCK-2R(−/−) mice. The *P* value between the values of histamine alone and histamine + carbachol did not reach the significant level ($P = 0.062$) in CCK-1R(−/−)2R(−/−) mice; however the value of histamine + carbachol was significantly higher than the value of carbachol alone.

Discussion

Gastric acid secretion in response to histamine and/or carbachol was quite different between mice with CCK-2R and mice without CCK-2R, as expected, because CCK-2R(−/−) mice [8, 9] as well as gastrin(−/−)CCK(−/−) mice [22] showed a decrease in parietal cell numbers.

The lowest dose (10 μg/kg) of carbachol produced significant increases in acid secretion in mice having CCK-2R [wild-type and CCK-1R(−/−) mice], but not in mice lacking CCK-2R [CCK-2R(−/−) and CCK-1R(−/−)2R(−/−) mice]. Thus, the sensitivity to carbachol in mice without CCK-2R was lower than that with CCK-2R. Moreover, the stimulatory effect of 50 μg/kg carbachol was most potent among the three doses, and significantly increased acid secretion in wild-type, CCK-1R(−/−), and CCK-1R(−/−)2R(−/−) mice, while no dose of carbachol produced a significant increase in CCK-2R(−/−) mice.

In previous reports [11, 20], somatostatin-producing D cells were shown to possess CCK-1R, and CCK to stimulate somatostatin release and synthesis. The present observation that administration of somatostatin abolished the response of acid secretion to carbachol in CCK-1R(−/−)2R(−/−) is compatible with the hypothesis that the lack of CCK-1R may result in a lack of tonic inhibition by endogenous somatostatin. The same mechanism may be applied to explain the rank of the mean values of basal acid secretion as follows: CCK-1R(−/−) > wild-type > CCK-1R(−/−)2R(−/−) > CCK-2R(−/−) mice. However, the response to histamine did not differ among four genotypes as described below. We speculate that the compensatory mechanism of histamine signaling pathway in CCK-2R(−/−) and 1R(−/−)2R(−/−) mice may exert more efficiently compared with carbachol pathway.

The responses of acid secretion to histamine were somewhat different from those to carbachol. The responses of gastric acid secretion stimulated by 0.1–1.0 mg/kg of histamine did not differ among four genotypes, although the

Table 2 Incremental gastric acid secretion (μmol/60 min per 10 g body weight) stimulated by 1.0 mg/kg histamine, 10 μg/kg carbachol, and 1.0 mg/kg histamine + 10 μg/kg carbachol

Genotype	Histamine alone	Carbachol alone	Histamine + carbachol
Wild-type	2.47 ± 0.40 (11)	5.76 ± 1.60 (9)	11.06 ± 1.88 (8)**
			$F(2,25) = 11.06$, $P < 0.0004$
CCK-1R(−/−)	1.75 ± 0.62 (6)	5.27 ± 1.05 (6)*	9.98 ± 1.24 (7)**
			$F(2,16) = 16.18$, $P < 0.0001$
CCK-2R(−/−)	2.12 ± 0.69 (8)*	0.31 ± 0.15 (7)	3.75 ± 0.29 (5)**
			$F(2,17) = 10.08$, $P < 0.0015$
CCK-1R(−/−)2R(−/−)	2.10 ± 0.77 (10)	0.37 ± 0.28 (6)	4.28 ± 1.02 (7)***
			$F(2,20) = 5.03$, $P < 0.017$

Values are means ± SE; numbers in the parentheses are the number of animals. The estimation methods are described in the text

* Significantly higher than the value of either histamine alone or carbachol alone, ** significantly higher than other two values, and *** significantly higher than the value produced by carbachol alone by the multiple comparison test

maximal response to the highest dose of histamine (10 mg/kg) remained at a significantly lower level in CCK-2R(−/−) mice than in wild-type mice. This is interpreted to mean that the sensitivity of parietal cells responding to exogenous histamine is conserved and that the sensitivity of the respective parietal cell should have been increased, although the maximal secretory capacity is limited in CCK-2R(−/−) mice. We could not examine the effect of 10 mg/kg histamine in CCK-1R(−/−) and CCK-1R(−/−)2R(−/−) mice, because these mice were not available at this time.

The reason why the responses to histamine and to carbachol were different is unknown. One possibility is that as histamine content in CCK-2R(−/−) mice was decreased due to impaired cellular differentiation by the lack of CCK-2R [8], H2-R function may be up-regulated or the number of H2-Rs may be increased to compensate for the decrease in histamine content. On the other hand, there is a report [23] that gastrin increased M3-R mRNA in sheep. High plasma concentrations of gastrin were observed in CCK-2R(−/−) mice [8, 10]; however, gastrin could not act because of the lack of its receptor. Therefore, the lack of gastrin action may have resulted in a decrease of M3-R expression in CCK-2R(−/−) and CCK-1R(−/−)2R(−/−) mice. The lack of response to 10 μg of carbachol in CCK-2R(−/−) and 1R(−/−)2R(−/−) mice may be attributable to decreases in the number of parietal cells as well as decreases in M3-R expression. The experiment using isolated parietal cells is necessary to elucidate precise mechanism of acid secretion in response to carbachol and histamine in these mice in the future.

It has been reported [21] that there is a potentiation effect of combined stimuli that increase either cellular cAMP or calcium, such as histamine and carbachol. Combined stimulation produced higher increases in acid secretion than the sums of values produced by either histamine or carbachol in each genotype. Therefore, it is suggested that the intracellular pathways of histamine and carbachol are maintained in mice without CCK-2R.

In conclusion, the presence of CCK-2R is important for maximal gastric acid secretion. The lack of CCK-2R alters sensitivity to carbachol but not to histamine. The biological role of CCK-1R in acid secretion seems to be less important.

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