Cholinergic agonist-induced pepsinogen secretion from murine gastric chief cells is mediated by M1 and M3 muscarinic receptors

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Xie, Guofeng, Cinthia Drachenberg, Masahisa Yamada, Jürgen Wess, and Jean-Pierre Raufman. Cholinergic agonist-induced pepsinogen secretion from murine gastric chief cells is mediated by M1 and M3 muscarinic receptors. Am J Physiol Gastrointest Liver Physiol 289: G521–G529, 2005. First published June 2, 2005; doi:10.1152/ajpgi.00105.2004.—Muscarinic cholinergic mechanisms play a key role in stimulating gastric pepsinogen secretion. Studies using antagonists suggested that the M3 receptor subtype (M3R) is involved in this activity. The development of a molecular approach using RT-PCR confirmed the expression of M3 receptors in chief cells. These data clearly indicate that, in gastric chief cells, a mixture of M1 and M3 receptors mediates cholinergic agonist-induced pepsinogen secretion. Pepsinogen secretion from murine gastric glands was determined by adapting methods used for rabbit and rat stomach. In wild-type (WT) mice, maximal concentrations of carbachol and CCK caused a 3.0- to 2.5-fold increase in pepsinogen secretion, respectively. Maximal carbachol-induced secretion from M3R−/− mouse gastric glands was decreased by 25%. In contrast, there was only a slight decrease in carbachol potency and no change in efficacy when comparing M3R−/− with WT glands. To explore the possibility that both M1R and M3R are involved in carbachol-mediated pepsinogen secretion, we examined secretion from glands prepared from M1αR−/− double-knockout mice. Strikingly, carbachol-induced pepsinogen secretion was nearly abolished in glands from M1αR−/− mice, whereas CCK-induced secretion was not altered. In situ hybridization for murine M1R and M3R mRNA in gastric mucosa from WT mice revealed abundant signals for both receptor subtypes in the cytoplasm of chief cells. These data clearly indicate that, in gastric chief cells, a mixture of M1R and M3R plays a key role in stimulating gastric pepsinogen secretion. Studies excluded the possibility that chief cells coexpress M1R and M3R.

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induced pepsinogen secretion, thereby facilitating studies with transgenic animals. Our findings demonstrate that, in gastric chief cells, a mixture of M1 and M3 receptors mediates cholinergic stimulation of pepsinogen secretion and that no other muscarinic receptor subtypes contribute to this activity. Moreover, dispersed gastric glands from mouse stomach can be used to evaluate the regulation of pepsinogen secretion in transgenic animals.

**EXPERIMENTAL PROCEDURES**

**Materials.** Collagenase (type I), BSA (fraction V), carbamylcholine (carbachol), vasoactive intestinal peptide (VIP), secretin, COOH-terminal octapeptide of CCK (CCK-8), phorbol 12-myristate 13-acetate (PMA), and A-23187 were from Sigma; MEM amino acids (50 times concentrated) and essential vitamin solution (100 times concentrated) were from Mediatech (Herndon, VA); and 125I-albumin was from ICN.

**Animals.** The generation of M1R−/−, M2R−/−, and M3R−/− mutant mice has been previously described (11). In all experiments, aged-matched WT mice of the corresponding genetic background had the following genetic background: 129S6/SvEvTac × CF1 (50/50%). The M2R−/− mice and the corresponding WT mice had a slightly different genetic background (129S4/SvJae × CF1; 50/50%; see Ref. 12). M1R−/− double-knockout (KO) mice (genetic background: 129S6/SvEvTac × CF1; 50/50%) were generated by intermating homozygous MIR−/− and M2R−/− mutant mice (11). In all experiments, aged-matched WT mice of the corresponding genetic background were used as control animals. Animals were genotyped by PCR analysis of mouse tail DNA. All experiments were carried out with adult male mice that were at least 3 mo old.

**Preparation of mouse gastric glands.** Gastric glands from mouse stomach were prepared by adapting methods used for rabbit (4) and rat stomach (28). Briefly, freshly separated and rinsed gastric mucosa was cut into several small pieces and placed in a flask containing 10 ml incubation solution and 0.1% (wt/vol) collagenase, gassed with O2, and incubated for 1 h at 37°C. Solutions were gassed with O2 for 30 s, and fresh digestion solution with collagenase (0.1%) was added, gassed at 150 oscillations/min. After discarding the digestion solution, 10 ml of glands from one stomach was filtered through a nylon mesh, centrifuged at 200 g for 30 s, and washed two times with fresh incubation solution. Glands were resuspended in 20 ml standard incubation solution. The final concentration of glands from one stomach was ~3 × 10⁶ glands/ml. Standard incubation solution contained 24.5 mM HEPES (adjusted to pH 7.4), 98 mM NaCl, 6 mM KCl, 2.5 mM KH₂PO₄, 1 mM MgCl₂, 11.5 mM glucose, 5 mM sodium pyruvate, 5 mM sodium fumarate, 5 mM sodium glutamate, 1.5 mM CaCl₂, 2 mM glutamine, 0.1% (wt/vol) BSA, 1% (wt/vol) amino acid mixture, and 1% (wt/vol) essential vitamin mixture. The standard incubation solution was equilibrated with 100% O₂, and all incubations were performed with 100% O₂ as the gas phase.

**Pepsinogen secretion.** Pepsinogen secretion was determined as previously described (27) using 125I-albumin as substrate and expressed as the percentage of total cellular pepsinogen that was released in the medium during the incubation. Histology. Stomachs from WT and KO mice were rinsed briefly in PBS and fixed in 10% neutral buffered formalin, processed by standard methods, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E) and toluidine blue (to highlight zymogen granules).

**Determination of pepsinogen content.** To measure pepsinogen content, gastric mucosa and gastric glands from WT and muscarinic receptor KO mice were sonicated in standard incubation solution using a Branson Digital Sonifier. Pepsinogen content was determined by converting pepsinogen to pepsin using the method of Anson and Minsky (3) as modified by Schlamowitz and Peterson (32) to measure tyrosine release from the hydrolysis of Hb (OD, 280 nm; Beckman Coulter DU530 spectrophotometer; see Refs. 28 and 31). Peptic activity was constant for at least 10 min at 37°C, and the assay was linear over a range that was twofold that of the maximal value assayed. Total protein was measured with the Quantipro BCA Assay Kit (Sigma). Porcine pepsinogen (3,350 pepstein units/mg protein; Sigma) was used as a standard to quantitate pepsinogen.

**RNA labeling.** Digoxigenin-labeled antisense RNA probes were prepared from riboprobe plasmids containing the M₁R and M₃R inserts. The M₁R riboprobe was synthesized from a 0.28 kb KpnI-SacI genomic fragment cloned into a pBluescript vector and corresponded to the M₁R sequence lacking in the genome of M₁R−/− mice (9). The M₃R riboprobe was synthesized from a 1.6 kb XhoI-SsrI genomic fragment corresponding to the M₃R sequence absent in the genome of M₃R−/− mice (39). The M₁R and M₃R riboprobes were digested with KpnI and NotI, respectively. After purification of the sonicated plasmids, in vitro transcriptions for M₁R and M₃R RNA probes, 280 bp and 1.6 kb in length, respectively, were performed using the Digoxigenin RNA labeling kit (Roche Applied Sciences) with T7 and T3 RNA polymerases, respectively. The length of the M₃R digoxigenin-labeled RNA was shortened by alkaline hydrolysis to ~300 bp. The yield of transcripts was estimated using dot blots with control digoxigenin-labeled RNA that was provided by Roche Applied Science.

**ISH.** The ISH procedure was adapted from the manual provided by Roche Applied Science (www.roche-applied-science.com). Stomachs from WT mice and muscarinic receptor KO mice were excised, washed briefly in PBS, and fixed overnight in 10% neutral buffered formalin. Paraffin blocks were prepared, and 5-μm sections were obtained. All mRNA hybridization steps and washes were carried out at 37°C, and posthybridization steps, except washing, were performed at room temperature. All materials were purchased from Roche Applied Science, except as noted. Paraffin sections were dewaxed and rehydrated before ISH. Slides were washed for 3 min with PBS and two times for 5 min each with 2× SSC buffer (3 M NaCl and 0.3 M sodium citrate, pH adjusted to 7.0). Samples were incubated with TE buffer (10 mM Tris·HCl, 1 mM EDTA, pH adjusted to 8.0) containing 2–5 μg/ml RNase-free proteinase K for 30 min and rinsed two times for 5 min each with PBS. Prehybridization was carried out with 400 μl hybridization buffer for 1 h. Samples were then incubated with 400 μl hybridization buffer containing 100 ng M₁R or M₃R digoxigenin-labeled RNA overnight. After hybridization, slides were washed two times for 15 min each with 2× SSC, two times for 15 min each with 1× SSC, and two times for 30 min each in 0.1× SSC. Samples were then incubated two times for 10 min each with buffer 1 and blocked for 30 min with buffer 1 containing 0.1% Triton X-100 and 1.5% sheep serum. Slides were then incubated for 2 h with 1:300 sheep anti-digoxigenin-alkaline phosphatase Fab fragments, washed two times for 10 min each with buffer 1 and again for 10 min with buffer 2. Samples were stained with 4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolylphosphate in the presence of 1 mM levamisole in buffer 2 for 2–3 h and washed briefly with TE and water before imaging.

**Statistical analysis.** Significance between two means was determined by Student’s t-test; P < 0.05 was considered significant. For multiple comparisons, Dunnett’s method was used together with least-squares means to provide appropriate corrections.

**RESULTS**

To prepare dispersed gastric glands from mouse stomach, we adapted methods used for rabbit (4) and rat (28) stomach (see EXPERIMENTAL PROCEDURES). Gastric glands obtained from WT and KO mice using this procedure were similar in shape (Fig. 1) and yield [6 ± 2 × 10⁵ (SE) glands/per stomach],
Likewise, the size and shape of murine gastric glands were similar to that previously described for glands from rat stomach (28).

To evaluate the utility of dispersed gastric glands from mouse stomach to study pepsinogen secretion, we compared the actions of a variety of secretagogues on pepsinogen secretion from WT murine gastric glands with that previously reported from rat gastric glands and guinea pig chief cells (Table 1; see Refs. 28 and 31). Secretagogues that stimulate pepsinogen secretion can be separated into two major categories: agents that activate adenylyl cyclase, thereby causing an increase in cellular cAMP, and agents that activate phospholipase C, thereby increasing cell calcium (25). Secretin, VIP, and histamine are agents in the former and carbachol (carbachol) and CCK are agents in the latter category. These agents interact with different classes of plasma membrane receptors (25). In addition, we tested the activity of the divalent cation ionophore, A-23187, which increases cell calcium concentration and a phorbol ester, PMA, which directly activates protein kinase C. A-23287 and PMA do not act by a plasma membrane receptor-mediated mechanism.

Table 1 shows maximal pepsinogen secretion from freshly dispersed mouse gastric glands observed after incubation with agents that activate adenylyl cyclase or phospholipase C. The table also compares the maximal secretory response for mouse glands with that observed previously using rat glands and guinea pig chief cells (28, 31). Overall, the magnitude of maximal secretory responses to these agents was comparable among the three species; an approximately threefold increase in pepsinogen release was observed compared with basal secretion from WT mouse gastric glands with that previously described for glands from rat stomach and guinea pig chief cells (28, 31). Although quantitative morphometric analysis was not performed, there was no apparent change in mucosal thickness or cell number. Likewise, there was no difference in the pepsinogen content of gastric mucosa obtained from WT, M1R−/−, M3R−/−, or M1/3R−/− mice (Table 2). Moreover, the pepsinogen content of dispersed gastric glands from WT and M1R−/− mice was not significantly different (Table 2).

To determine the importance of M1-M3 receptors for cholinergic agonist-induced pepsinogen release, we compared carbachol dose-response curves from dispersed gastric glands prepared from age-matched WT, M1R−/−, M3R−/−, M1R−/−, and M1/3R−/− mice (Table 2). Maximal secretagogue-induced pepsinogen secretion from mouse and rat gastric glands and from dispersed chief cells prepared from guinea pig stomach (28, 31, 34).

Table 1. Maximal secretagogue-induced pepsinogen secretion from mouse and rat gastric glands and from dispersed chief cells prepared from guinea pig stomach

<table>
<thead>
<tr>
<th>Secretagogue</th>
<th>Mouse gastric glands</th>
<th>Rat gastric glands*</th>
<th>Guinea pig chief cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.8 ± 0.5</td>
<td>2.7 ± 0.5</td>
<td>2.2 ± 0.4</td>
</tr>
<tr>
<td>Secretin (100 nM)</td>
<td>12.1 ± 1.0†</td>
<td>13.9 ± 1.0</td>
<td>11.3 ± 0.9</td>
</tr>
<tr>
<td>VIP (300 nM)</td>
<td>9.1 ± 1.2†</td>
<td>8.1 ± 2.1</td>
<td>7.2 ± 0.6</td>
</tr>
<tr>
<td>Histamine (10 μM)</td>
<td>6.6 ± 0.7†</td>
<td>ND</td>
<td>6.2 ± 1.2</td>
</tr>
<tr>
<td>Carbachol (100 μM)</td>
<td>12.6 ± 0.6†</td>
<td>13.0 ± 1.7</td>
<td>11.4 ± 0.8</td>
</tr>
<tr>
<td>CCK-8 (10 nM)</td>
<td>10.4 ± 0.8†</td>
<td>11.2 ± 1.3</td>
<td>9.0 ± 1.1</td>
</tr>
<tr>
<td>A-23187 (1 μM)</td>
<td>12.5 ± 1.9†</td>
<td>8.6 ± 2.1</td>
<td>12.5 ± 1.2</td>
</tr>
<tr>
<td>PMA (100 nM)</td>
<td>12.3 ± 1.5†</td>
<td>ND</td>
<td>7.9 ± 0.7</td>
</tr>
</tbody>
</table>

In each experiment each value was determined in duplicate, and results given are means ± SE (mouse glands) or SD (rat glands and guinea pig chief cells) from at least 4 separate experiments. VIP, vasoactive intestinal peptide; PMA, phorbol 12-myristate 13-acetate. Dispersed gastric glands were incubated with the agents indicated at 37°C for 15 min. *Data for rat gastric glands and guinea pig chief cells were taken from Refs. 28 and 31 respectively. †Values significantly greater than basal (P < 0.05, Student’s t-test). ND indicates that no data are available.
The M2 muscarinic receptor is abundantly expressed in the gastrointestinal tract (primarily in smooth muscle; see Ref. 8). Although M2 receptors are generally coupled to inhibition of adenylyl cyclase activity (37), it has been reported that, on occasion, M2 receptors can be coupled to activation of phospholipase C-β (17). We therefore speculated that M2 receptors could play a role, at least theoretically, in carbachol-mediated pepsinogen release. However, as observed in glands from M3R/−/− mice, carbachol-induced pepsinogen secretion was the same when comparing the response in glands from WT and M2R/−/− mice (data not shown).

As shown in Fig. 3 and Table 3, basal pepsinogen secretion was the same in gastric glands prepared from WT and M1R/−/− mice. However, in contrast to the findings obtained with glands

### Table 2. Pepsinogen content of gastric mucosa and dispersed gastric glands from WT and muscarinic receptor knockout mice

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Pepsinogen Content, µg/mg protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
</tr>
<tr>
<td>Gastric mucosa</td>
<td>32.9±5.2</td>
</tr>
<tr>
<td>Gastric glands</td>
<td>22.3±1.7</td>
</tr>
</tbody>
</table>

Values are means ± SE. WT, wild type; M1R/−/−, M1 receptor deficient; M3R/−/−, M3 receptor deficient; M1/3R/−/−, M1 and M3 receptor deficient. To release pepsinogen from tissue, gastric mucosa and glands were sonicated in standard incubation solution. Total protein was measured using the QuantiPro BCA Assay Kit (Sigma). Pepsinogen content was determined by converting pepsinogen to pepsin and measuring the hydrolysis of hemoglobin (31). Porcine pepsinogen (3,350 peptic units/mg protein) was used as a standard to quantify pepsinogen in the samples. ND indicates that indicated values were not determined. NS indicates that, for both gastric mucosa and dispersed gastric glands, there was no significant difference (p > 0.05) when comparing results from tissue from WT animals with those from muscarinic receptor knockout animals.
from M1R−/− and M3R−/− mice, loss of the M1 receptor decreased the efficacy of carbachol-induced pepsinogen secretion by ~25% (Fig. 3 and Table 3), indicating that the M1 receptor plays a role in mediating cholinergic agonist-induced pepsinogen secretion. However, since a pronounced secretory response remained in glands from M1R−/− mice, we concluded that non-M1 muscarinic receptors must also be involved in this activity.

To test the hypothesis that the secretory response remaining in glands from M1R−/− mice is mediated by M3 receptors, we examined carbachol-induced pepsinogen secretion from M1/3R−/− double-KO mice. As shown in Fig. 3 and Table 3, loss of both M1 and M3 receptors essentially abolished carbachol-induced pepsinogen secretion. Pepsinogen secretion from gastric glands prepared from M1/3R−/− mice was not significantly different from basal pepsinogen release at any of the carbachol concentrations tested (Fig. 3).

To verify the integrity of the secretory machinery in chief cells from M1R−/− mice, we also examined the secretory actions of CCK-8. CCK interacts with CCK1 receptors, a different class of chief cell plasma membrane receptors that couple to similar G proteins (Gq class) as the M1 and M3 muscarinic receptors (25). In gland preparations from both WT and M1/3R−/− mice, pepsinogen secretion was detected with 10 pM and maximal with 1 nM CCK-8. As shown in Fig. 4 and Table 3, the concentration-response curves for CCK-8-induced pepsinogen secretion from gastric glands prepared from M1/3R−/− and WT mice were nearly indistinguishable. Likewise, there was no difference when comparing the potency or efficacy of CCK-8-induced pepsinogen secretion from WT or M1/3R−/− mice with that from guinea pig chief cells (Table 3 and Ref. 25).

To confirm that WT mouse chief cells express both M1R and M3R mRNA, we prepared riboprobes to detect the respective receptor mRNAs by ISH (see experimental procedures). As shown in Fig. 5A, with the M1R mRNA probe, ISH revealed abundant signal in the base of gastric mucosal sections prepared from WT and M3R−/− animals but not in sections prepared from M1R−/− and M1/3R−/− mice. Likewise, with the M3R mRNA probe, ISH revealed abundant signal in the base of gastric mucosal sections prepared from WT and M1R−/− animals but not in sections prepared from M1R−/− and M1/3R−/− mice (Fig. 5A). These findings confirm coexpression of M1R and M3R in chief cells in the base of gastric glands in WT mice and absence of muscarinic receptor subtype expression in gastric mucosa in the appropriate KO animals. Whereas expression of M1R appeared to be confined to chief cells at the base of gastric glands, cytoplasmic M3R mRNA hybridization signal was also noted in other cell types located primarily at the apex of the glands (Fig. 5A). Identification of these cells was beyond the scope of the present work. In parietal cells, we detected robust nuclear staining with the M3R riboprobe (Fig. 5A) consistent with previous observations regarding M3R expression in parietal cells (1, 16).

To more clearly define that WT mouse chief cells express both M1R and M3R mRNA, we examined ISH staining with M1R and M3R riboprobes of contiguous 5-μm gastric mucosal sections prepared from WT animals. As shown in Fig. 5B, with both riboprobes, abundant hybridization signal was obtained in cells having the morphological appearance of chief cells at the base of gastric glands. In these sections, all cells having the morphological characteristics of gastric chief cells (31) were stained by both the M1R and M3R riboprobes.

Collectively, these findings indicate that both M1 and M3 muscarinic receptors are involved in mediating carbachol-induced pepsinogen secretion, and both receptor subtypes must be eliminated to extinguish cholinergic agonist-induced pepsinogen secretion. The presence of either of these two receptor subtypes (M1R or M3R) alone is sufficient to permit robust carbachol-induced pepsinogen secretion responses.

**DISCUSSION**

Cholinergic agonists are potent and efficacious stimulators of pepsinogen secretion from mammalian gastric chief cells (25),

**Table 3. Comparison of basal and carbachol- and CCK-induced pepsinogen secretion from dispersed gastric glands prepared from WT, M1R−/−, M3R−/−, and M1/3R−/− mice**

<table>
<thead>
<tr>
<th>Muscarinic Receptor Status</th>
<th>Carbachol-induced Secretion</th>
<th>CCK-induced Secretion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>EC50, μM</td>
<td>Maximum, % total</td>
</tr>
<tr>
<td>WT</td>
<td>4.8 ± 0.6</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>M1R−/−</td>
<td>4.5 ± 0.3</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>M3R−/−</td>
<td>4.5 ± 0.4</td>
<td>4.1 ± 0.9</td>
</tr>
<tr>
<td>M1/3R−/−</td>
<td>4.2 ± 0.4</td>
<td>1.3 ± 0.2</td>
</tr>
</tbody>
</table>

Values are means ± SE. Gastric glands (3 × 10⁴ glands/ml) from WT and knockout mice were suspended in standard incubation solution containing the indicated concentrations of carbachol and incubated at 37°C for 15 min. Pepsinogen secretion was calculated as the percentage of pepsinogen in glands at the beginning of incubation that was released in the extracellular medium during incubation. In each experiment, each value was determined in duplicate, and results given are means from at least 4 separate experiments. EC50, the concentration of agonist that caused half-maximal pepsinogen secretion, was calculated using BioDataFit version 1.2 (Chang Bioscience); ND, not determined. *Values that were significantly less (P < 0.05, Student’s t-test) than those observed with gastric glands from WT animals.
Fig. 5. A: In situ mRNA hybridization for 
M₁R (row on top) and M₃R (row on bottom) using gastric mucosal sections prepared from WT, M₁R⁻/⁻, M₃R⁻/⁻, and M₁/₃R⁻/⁻ mice. Brackets indicate chief cells at the base of gastric glands. Green arrows indicate nuclear hybridization signals in parietal cells. B: high-magnification images from in situ mRNA hybridization with M₁R (left) and M₃R (right) probes using contiguous 5-μm gastric mucosal sections prepared from WT animals. Size bars = 100 μm. Images are representative of results observed in 3 separate animals.
Studies from our laboratory and others have demonstrated that ACh and other cholinergic agents, like carbachol, stimulate pepsinogen secretion in intact animals and in vitro models using dispersed gastric glands and isolated chief cells (28, 31). Atropine and certain subtype-selective muscarinic antagonists inhibit cholinergic agonist-induced pepsinogen secretion (34). However, pharmacological studies using receptor subtype-prefering muscarinic antagonists to identify the functional roles of individual muscarinic receptor subtypes have to be interpreted with caution, primarily because of the limited receptor subtype selectivity of these agents (6, 37).

Using RT-PCR, one group of investigators detected M3 muscarinic receptor mRNA in chief cells from rabbit stomach (16). A subsequent report from a different laboratory using ISH indicated that the M1 receptor is expressed abundantly in rat chief cells (13). It is therefore likely that M1R and M3R receptors are both expressed by gastric chief cells. To assess the potential functional roles of these receptor subtypes in cholinergic agonist-induced pepsinogen secretion, we took advantage of the availability of recently developed muscarinic receptor KO mice (18). To our knowledge, this is the first study to examine stimulated pepsinogen secretion in a gene KO mouse model. Aihara et al. (1) reported that basal pepsinogen secretion in the pylorus-ligated stomach was the same in WT and M1R−/− mice but did not evaluate stimulated secretion.

It is of interest to compare morphological and functional findings in muscarinic KO mice with those reported for the stomachs of CCK2R−/− and histamine2R−/− mice. CCK2R−/− mice showed a decreased thickness of the glandular mucosa and a decrease in the number of gastric parietal and enterochromaffin-like (ECL) cells (20, 23). Similar findings were obtained with mice deficient in gastrin, the primary CCK2R receptor ligand (10, 19). In contrast, disruption of the gene for histamine2R resulted in increased thickness of the glandular mucosa with an increased number of ECL and parietal cells (18). In the present study, no changes in mucosal thickness or cell number or distribution were appreciated after disruption of the different muscarinic receptor genes. With regard to the M3R−/− mouse, our findings are in accord with those of Aihara et al. (1), who also observed no alteration in mucosal thickness or cell distribution in the gastric mucosa of the KO animals. These findings may reflect the observation that gastrin, unlike cholinergic agonists, functions as a necessary trophic factor for gastrointestinal tissues (15).

Carbachol-induced pepsinogen secretion from M3R−/− and M2R−/− mouse glands was the same as that from WT mouse glands. In contrast, secretion from M1R−/− mouse glands was significantly reduced (by ~25%) compared with that from WT glands. However, a pronounced secretory response remained in glands from M1R−/− mice (~75% of the maximal response of WT preparations). Strikingly, carbachol-induced pepsinogen secretion was abolished in mice deficient in both M1R and M3R receptors. These findings suggest that a mixture of M1R and M3R muscarinic receptors mediate muscarinic agonist-induced pepsinogen secretion and that no other muscarinic receptor subtype makes a significant contribution to this activity. Our data are consistent with the concept that gastric chief cells express both M1R and M3R muscarinic receptors and that the presence of either M1R or M3R muscarinic receptors is sufficient to allow for robust muscarinic agonist-induced pepsinogen secretion, indicative of a high degree of “receptor reserve.” Coexpression, per se, of muscarinic receptor subtypes on a single cell type is not a novel finding. For example, previous studies have indicated coexpression of M1R and M3R receptors in rat striatum (36) and sublingual glands (7). Moreover, in rat pancreatic acinar cells that have receptors and signal transduction pathways very similar to those described for gastric chief cells (25), pharmacological and PCR analysis was consistent with the presence of both M1R and M3R receptors (33).

In contrast to preparations of isolated chief cells, gastric glands contain a heterogeneous cell population that includes chief, parietal, mucous, ECL, and other cell types. Our findings with KO mice raise the question of whether cholinergic agonist-induced pepsinogen secretion is mediated by M1R or M3R receptors coexpressed on chief cells or whether an intermediary cell plays a role in regulating this activity. Because of the proximity of ECL and chief cells, it is possible, at least theoretically, that, as with gastric parietal cells (14), ECL cells may regulate chief cell function. ECL cell regulation of parietal cell function is mediated by paracrine effects of histamine that is released from the ECL cell and interacts with histamine2R on parietal cells (14).

Our experiments examining the secretory effects of histamine on murine gastric glands are germane to addressing the possibility that histamine released as a consequence of muscarinic stimulation of ECL or other gastric mucosal cells contributes to maximal carbachol-induced pepsinogen secretion. As shown in Table 1, the maximal secretory response to histamine was modest, with pepsinogen secretion only slightly greater than basal. Moreover, the maximal secretory response to histamine was ~50% of the maximal response observed when glands from M1R−/− mice were treated with carbachol. This observation indicates that the secretory response to maximal concentrations of histamine is insufficient to account for maximal carbachol-induced secretion in gastric glands from M1R−/− mice.

Our ISH studies using specific riboprobes for M1R and M3R mRNA provide more direct evidence that murine gastric chief cells coexpress M1R and M3R muscarinic receptor subtypes. It is of interest that these studies demonstrate expression of M1R mRNA only in chief cells, whereas M3R mRNA is expressed in other cell types in the murine gastric gland (Fig. 5A). Although it was beyond the scope of the present studies to explore the nature of the other cells in the gastric gland that express M3R, our finding of reproducible M3R mRNA hybridization signal in the nuclei of gastric parietal cells is compatible with reports that these cells express M3 muscarinic receptors (1). The explanation for the greater cytoplasmic signal with M3R riboprobe in the cytoplasm of chief cells compared with parietal cells is not readily apparent. Nevertheless, in support of the objective of the present work, it is apparent from these ISH experiments that, in mice, gastric chief cells have particularly high levels of M3R mRNA expression.

Based on our findings and those of others (13, 16, 34), we conclude that gastric chief cells coexpress M1R and M3R muscarinic receptors, both of which can stimulate profound pepsinogen secretion independently of each other. M1R receptors appear to be functionally more important, since their absence causes a 25% decrease in maximal carbachol-induced pepsinogen secretion, whereas there is no appreciable difference in carbachol activity when M3R receptors are knocked out. Given the importance of vagal gastric stimulation in digestive physiology, the
presence of two functionally related muscarinic receptor subtypes on gastric chief cells may provide an evolutionary advantage in the event of mutational changes that inactivate one class of receptors. We cannot exclude the possibility that, in the chief cell, M1 and M3 receptors couple to additional functions that are unrelated to pepsinogen secretion, nor can we exclude the possibility that, in vivo, other muscarinic receptor subtypes are involved (e.g., M5 receptors in the murine gastric submucosal plexus; see Ref. 2).

In conclusion, the data presented here indicate the utility of muscarinic receptor KO mice to identify the muscarinic receptor subtypes involved in mediating pepsinogen secretion. Using these animal models and ISH, we demonstrated unambiguously that a mixture of M1 and M3 muscarinic receptors mediates muscarinic agonist-induced pepsinogen secretion and that no other muscarinic receptor subtype makes a significant contribution to this activity. The presence of either M1 or M3 receptors is sufficient to allow for robust muscarinic agonist-induced pepsinogen secretion. Finally, the secretory responses of dispersed gastric glands prepared from mouse stomach to a variety of stimulants is similar to those reported for rat glands and guinea pig chief cells. The development of this murine secretory model will facilitate the use of transgenic mice to investigate the regulation of pepsinogen secretion by noncholinergic mechanisms.

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