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

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Submitted 9 March 2004; accepted in final form 23 May 2005

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 M₁ receptor subtype (M₁R) plays a prominent role in mediating pepsinogen secretion, but in situ hybridization indicated expression of M₁ receptor (M₁R) in rat chief cells. We used mice that were deficient in either the M₁ (M₁R−/−) or M₃ (M₃R−/−) receptor or that lacked both receptors (M₁R−/−M₃R−/−) to determine the role of M₁R and M₃R in mediating 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- and 2.5-fold increase in pepsinogen secretion, respectively. Maximal carbachol-induced secretion from M₁R−/− 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 M₁R−/−M₃R−/− double-knockout mice. Strikingly, carbachol-induced pepsinogen secretion was nearly abolished in glands from M₁R−/−M₃R−/− 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 M₁ and M₃ receptors mediates cholinergic stimulation of pepsinogen secretion and that no other muscarinic receptor subtypes are involved in this activity. The development of a murine secretory model facilitates use of transgenic mice to investigate the regulation of pepsinogen secretion.

Studies using animal and tissue models have identified a variety of agents that stimulate gastric pepsinogen secretion. Stimulation by the vagus nerve, which is mediated by release of the neurotransmitter ACh, is probably the most important physiological mechanism for increasing pepsinogen release in the gastric lumen during the digestive process (see Ref. 26 for review of the regulation of pepsinogen secretion). Cholinergic stimulation of gastric acid and pepsinogen secretion also contributes to the pathogenesis of stomach and duodenal ulcers, and surgical vagotomy and the use of anticholinergic agents have been important modes of therapy for these conditions (26). Cholinergic agonists like ACh interact with muscarinic receptors on chief cells, thereby causing activation of phospholipase C and production of second messengers (28, 31). In turn, these second messengers cause an increase in intracellular calcium concentration (27, 35) that activates protein kinases and phosphatases, ultimately resulting in pepsinogen release (24, 29, 30).

Molecular cloning studies revealed the existence of five muscarinic receptor genes, designated M₁–M₅ (37, 38). Of these five receptor subtypes, types M₁, M₃, and M₅ are coupled to G proteins of the G₁ family, whereas types M₂ and M₄ are coupled to G₂-class G proteins (37). M₁ and M₃ receptors are expressed in many exocrine glands, whereas M₅ receptors are expressed only at low levels in the brain and other tissues (5, 21, 37). There is controversy regarding which muscarinic receptor subtype mediates cholinergic agonist-induced gastric pepsinogen secretion. Pharmacological studies in guinea pig chief cells using subtype-prefering muscarinic antagonists suggested that the M₃ receptor (formerly designated M₂c) mediates pepsinogen secretion (34). In rabbit gastric glands, a molecular approach using RT-PCR confirmed the expression of M₃ receptors in chief cells (16). A subsequent in situ hybridization (ISH) study indicated that rat chief cells abundantly express M₁ receptors (13). However, none of these studies excluded the possibility that chief cells coexpress M₁ and M₃ receptors. For example, pharmacological and PCR analysis of muscarinic receptor expression in dispersed acini from rat pancreas are consistent with expression of both M₁ and M₃ muscarinic receptors (33). It is of interest that the cellular biology of the gastric chief cell and pancreatic acinar cell is very similar (25).

Recently, mutant mouse strains deficient in individual muscarinic receptors have become available as novel experimental tools (38). In this study, we used several of these mutant mouse strains to determine which muscarinic receptor subtype(s) are required to mediate cholinergic agonist-induced pepsinogen secretion. Specifically, we analyzed mice that were deficient in either the M₁ (M₁R−/− mice) or M₃ (M₃R−/− mice) receptor subtype or that lacked both the M₁ and M₃ receptor subtypes (M₁R−/−M₃R−/− mice). For control purposes, we also analyzed mice deficient in M₂ receptors (M₂R−/− mice). A secondary objective was to develop a preparation of dispersed glands from mouse stomach that can be used to examine secretagogue-
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 acetate (PMA), and A-23187 were from Sigma; MEM amino acids was from ICN.

Animals. The generation of M1R<sup>+/−</sup>, M3R<sup>−/−</sup>, and M1R<sup>−/−</sup>, M3R<sup>−/−</sup> mutant mice has been described previously (9, 12, 22, 39). The M1R<sup>+/−</sup> and M1R<sup>−/−</sup> and the corresponding wild-type (WT) mice had the following genetic background: 129S6/SvEvTac·CF1 (50/50%). The M1R<sup>+/−</sup> mice and the corresponding WT mice had a slightly different genetic background (129S4/SvJae·CF1; 50/50%; see Ref. 12). M3R<sup>−/−</sup> double-knockout (KO) mice (genetic background: 129S6/SvEvTac·CF1; 50/50%) were generated by interbreeding homozygous M1R<sup>−/−</sup> and M3R<sup>−/−</sup> mutant mice (11). In all experiments, age-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 100% O<sub>2</sub>, and all incubations were performed with 100% O<sub>2</sub> as the gas phase.

Histology. Stomachs from WT and KO mice were rinsed briefly in PBS and fixed in 10% neutral buffered formalin, processed by the following genetic background: 129S6/SvEvTac·CF1; 50/50%; see Ref. 27) using 125I-albumin as substrate and excised. Total protein was measured with the Quantiprobe BCA Assay Kit (Sigma). Porcine pepsinogen (3,350 peptic 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-BglII genomic fragment cloned into a pBluescript vector and corresponded to the M1R sequence lacking in the genome of M1R<sup>−/−</sup> mice (9). The M3R riboprobe was synthesized from a 1.6-kb XhoI-SnaI genomic fragment corresponding to the M3R sequence absent in the genome of M1R<sup>−/−</sup> mice (39). The M1R and M3R riboprobes were digested with KpnI and NotI, respectively. After purification of the isolated 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 M1R 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 (0.3 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 twice 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 twice 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 twice 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 twice 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 × 10<sup>5</sup> (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 adenyl 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 carbamylcholine (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 adenyl 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 values (Table 1). As seen previously in the rat and guinea pig preparations, maximal responses to VIP and histamine were of lower magnitude than those of the other agents tested. These findings indicate that the secretory responses obtained with this newly developed method for preparing gastric glands from murine stomach are robust and of sufficient magnitude for studying the regulation of pepsinogen secretion.

Disruption of the CCK2 (previously designated CCK-B or gastrin) and histamine type-2 receptor genes in mice led to changes in gastric glandular morphology (18, 20, 23). To determine whether the loss of either M1 or M3 muscarinic receptors or the simultaneous loss of both M1 and M3 receptors affected the morphology of the gastric glands and chief cells, we performed H&E staining on cross sections of stomachs from WT and muscarinic receptor KO mice. H&E staining and low- and high-power microscopic examination showed that the gastric glandular morphology was the same when comparing gastric tissues obtained from M1R<sup>-/-</sup>, M3R<sup>-/-</sup>, and M1/3R<sup>-/-</sup> mice with those from WT mice (Fig. 2). Moreover, chief cells were located in their normal position at the base of gastric glands, and cellular polarity with the nucleus at the basal pole and zymogen granules at the apical pole was preserved (Fig. 2; see Ref. 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<sup>-/-</sup>, M3R<sup>-/-</sup>, or M1/3R<sup>-/-</sup> mice (Table 2). Moreover, the pepsinogen content of dispersed gastric glands from WT and M1/3R<sup>-/-</sup> 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<sup>-/-</sup>, M3R<sup>-/-</sup>, M1/3R<sup>-/-</sup>, and M1/3R<sup>-/-</sup> mice (Table 2). Moreover, the pepsinogen content of dispersed gastric glands from WT and M1/3R<sup>-/-</sup> mice was not significantly different (Table 2).

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

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<tr>
<th>Secretagogue</th>
<th>Maximal Pepsinogen Secretion, %/total</th>
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<td></td>
<td>(Mouse gastric glands)</td>
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<tr>
<td>None</td>
<td>3.8 ± 0.5</td>
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<tr>
<td>Secretin (100 nM)</td>
<td>12.1 ± 1.0&lt;sup&gt;†&lt;/sup&gt;</td>
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<tr>
<td>VIP (300 nM)</td>
<td>9.1 ± 1.2&lt;sup&gt;†&lt;/sup&gt;</td>
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<tr>
<td>Histamine (10 μM)</td>
<td>6.6 ± 0.7&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>Carbachol (100 μM)</td>
<td>12.6 ± 0.6&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>CCK-8 (100 nM)</td>
<td>10.4 ± 0.8&lt;sup&gt;†&lt;/sup&gt;</td>
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<tr>
<td>A-23187 (1 μM)</td>
<td>12.5 ± 1.9&lt;sup&gt;†&lt;/sup&gt;</td>
</tr>
<tr>
<td>PMA (100 nM)</td>
<td>12.3 ± 1.5&lt;sup&gt;†&lt;/sup&gt;</td>
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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 adenyl 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

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<th>Table 2. Pepsinogen content of gastric mucosa and dispersed gastric glands from WT and muscarinic receptor knockout mice</th>
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<td>Preparation</td>
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<td>Gastric mucosa</td>
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<td>Gastric glands</td>
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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 M1/3R−/− 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 M1/3R−/− and M1R−/− 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 stimulants of pepsinogen secretion from mammalian gastric chief cells (25).
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<sup>−/−</sup>, M3R<sup>−/−</sup>, and M1/3R<sup>−/−</sup> 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 M1 and M3 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 (38). 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 M3R−/− mice but did not evaluate stimulated secretion.

It is of interest to compare morphological and functional findings in murine gastric 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 CCK2 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 (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 M1 and M3 muscarinic receptors, both of which can stimulate profound pepsinogen secretion independently of each other. M1 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 M3 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 inactive 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.

ACKNOWLEDGMENTS

We thank Jennifer James, Department of Pathology, for embedding, sectioning, and staining mouse gastric sections and Drs. Terry Sims and Fengjuan Zhang from the Departments of Anatomy and Pathology, respectively, for use of microscopes and cameras (all at the University of Arkansas for Medical Sciences). We are also grateful to Yinghong Cui, Laboratory of Bioorganic Chemistry, National Institutes of Health, for assistance in preparation of the riboprobes and for excellent technical assistance during the generation of the KO mice.

GRANTS

This work was supported by the Office of Research and Development, Medical Research Service, Department of Veterans Affairs and Biomedical Research Foundation, Central Arkansas Veterans Health Care System.

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AJP-Gastrointest Liver Physiol • VOL 289 • SEPTEMBER 2005 • www.ajpgi.org


