Cholinergic Stimulation of Amylase Secretion from Pancreatic Acinar Cells Studied with Muscarinic Acetylcholine Receptor Mutant Mice

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ABSTRACT

Muscarinic acetylcholine receptors (mAChRs) expressed by pancreatic acinar cells play an important role in mediating acetylcholine-dependent stimulation of digestive enzyme secretion. To examine the potential roles of M1 and M3 mAChRs in this activity, we used M1 and M3 receptor single knockout (KO) and M1/M3 receptor double KO mice as novel experimental tools. Specifically, we examined the ability of the muscarinic agonist carbachol to stimulate amylase secretion in vitro, using dispersed pancreatic acini prepared from wild-type and mAChR mutant mice. Quantitative reverse transcription-polymerase chain reaction studies using RNA prepared from mouse pancreatic acini showed that deletion of the M1 or M3 mAChR genes did not lead to significantly altered mRNA levels of the remaining mAChR subtypes. Moreover, immunoprecipitation studies with M1 and M3 mAChR-selective antisera demonstrated that both mAChR subtypes are expressed by mouse pancreatic acini. Strikingly, carbachol-induced stimulation of amylase secretion was significantly impaired in acinar preparations from both M1 and M3 receptor single KO mice and abolished in acinar preparations from M1/M3 receptor double KO mice. However, another pancreatic secretagogue, bombesin, retained its ability to fully stimulate amylase secretion in acinar preparations from M1/M3 receptor double KO mice. Together, these studies support the concept that cholinergic stimulation of pancreatic amylase secretion is mediated by a mixture of M1 and M3 mAChRs and that other mAChR subtypes do not make a significant contribution to this activity. These findings clarify the long-standing question regarding the molecular nature of the mAChR subtypes mediating the secretion of digestive enzymes from the exocrine pancreas.

The function of the exocrine pancreas, the major site of production of digestive enzymes, is under both neuronal and humoral control (Gardner and Jensen, 1993; Holst, 1993; Chey and Chang, 2001). The extrinsic parasympathetic (vagal) innervation of the pancreas plays a major role in stimulating enzyme secretion from the exocrine pancreas (Holst, 1993). After the stimulation of pancreatic parasympathetic nerves (e.g., after food intake), acetylcholine (ACh) is released from vagal (cholinergic) nerve terminals and interacts with muscarinic ACh receptors (mAChRs) located directly on pancreatic acinar cells to stimulate enzyme secretion (Gardner and Jensen, 1993). Interestingly, muscarinic cholinergic mechanisms may also contribute to the pathogenesis of certain forms of pancreatitis (Marsh et al., 1988; Possani et al., 1991; Grönroos et al., 1992).

Identification of the specific mAChR subtype(s) involved in stimulating pancreatic digestive enzyme secretion is required for better understanding exocrine pancreas function. Molecular cloning studies have shown that the mAChR family consists of five molecularly distinct members (M1–M5; Wess, 1996; Caulfield and Birdsell, 1998). The M1, M3, and M5 receptors are selectively coupled to G proteins of the Gq family, which mediate the breakdown of phosphatidyl inositol lipids, whereas the M2 and M4 receptors are preferentially linked to G proteins of the Gi class, which, at a biochemical level, mediate the inhibition of adenylyl cyclase (Wess, 1996; Caulfield and Birdsell, 1998).

In the past, several groups have reported that ACh (muscarinic agonist)-induced stimulation of exocrine pancreas secretion is mediated by the M3 receptor subtype (Louie and
Owyang, 1986; Kore et al., 1987; Iwatsuki et al., 1989; van Zwam et al., 1990; Kato et al., 1992; Love et al., 1999). This conclusion was based on radioligand binding and functional studies using several subtype-prefering muscarinic antagonists, such as pirenzepine (preferentially binds to M₁ receptors), AF-DX 116 (methoctramine, preferentially binds to M₂ and M₄ receptors), and 4-DAMP (~10 higher affinity for M₁, M₃, M₄, and M₅ receptors than for M₂ receptors) (Dörje et al., 1991; Wess, 1996; Caulfield and Birdsell, 1998). However, the subtype “selectivity” of these antagonists, like that of most other subtype-prefering muscarinic antagonists currently used as pharmacological tools, is limited (Wess, 1996; Caulfield and Birdsell, 1998). The possibility therefore exists that other mAChR subtypes, which may be less abundant than the M₅ receptor subtype, may also contribute to mAChR-mediated stimulation of pancreatic enzyme secretion but may have remained undetected in previous pharmacological studies.

To circumvent the difficulties involved with the use of muscarinic antagonists endowed with a limited degree of receptor subtype selectivity, we used gene targeting technology to generate mice lacking functional M₁, M₂, M₃, M₄, or M₅ mAChRs (for review, see Wess, 2004). We recently also generated a series of mAChR double KO mice (Duttaroy et al., 2002; Struckmann et al., 2003; Gautam et al., 2004). During the past few years, many studies have shown that these mutant animals are highly useful new tools for delineating the physiological and/or pathophysiological roles of the individual mAChR subtypes (Wess, 2004).

Interestingly, recent RT-PCR studies have shown that rat pancreatic acini not only express M₅ but also M₁ mAChR mRNA (Schmid et al., 1998; Turner et al., 2001). To examine whether both acinar M₁ and M₅ receptors play a role in cholinergic stimulation of exocrine pancreas secretion, we used M₁ (Miyakawa et al., 2001; Fisahn et al., 2002) and M₅ receptor single KO mice (Yamada et al., 2001) and recently generated M₁/M₅ receptor double KO mice (Gautam et al., 2004) as novel experimental tools. Specifically, we examined the ability of the muscarinic agonist carbachol to stimulate amylase secretion in vitro, using dispersed pancreatic acini prepared from wild-type (WT) and mAChR mutant mice. We also studied mAChR receptor expression levels in mouse pancreatic acini using TaqMan RT-PCR (Gautam et al., 2004) and immunoprecipitation strategies (Miyakawa et al., 2001; Yamada et al., 2001; Fisahn et al., 2002).

We found that carbachol-induced stimulation of pancreatic amylase secretion was significantly reduced in acinar preparations from both M₁ and M₅ receptor single KO mice and abolished in acinar preparations from M₁/M₅ receptor double KO mice. These data indicate that mAChR-mediated stimulation of amylase secretion from the exocrine pancreas is mediated by a mixture of M₁ and M₅ mAChRs and that other mAChRs do not contribute to this activity to a significant extent. These findings clarify the long-standing question as to which mAChR subtypes play a role in regulating the secretory function of the exocrine pancreas.

Materials and Methods

Materials. Unless noted otherwise, all chemicals, including carbachol (carbamoyl chloride) and bombesin, were purchased from Sigma-Aldrich (St. Louis, MO). Collagenase was obtained from Crescent Chemicals (Islandia, NY) (catalog no. 1745N). [³H]Quinuclidinyl benzilate ([³H]QNB; specific activity 42 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Protein A Sepharose CL-4B beads were obtained from Amersham Biosciences Inc. (Piscataway, NJ).

Amylose Release from Dispersed Mouse Pancreatic Acini in Vitro. Amylase release from dispersed mouse pancreatic acini was measured using a procedure similar to that described by Huang et al. (1990), with some modifications. After removal of the preincubation medium, acini prepared from the pancreas of one mouse were suspended in 50 ml of fresh standard incubation buffer and preincubated for 15 min at 37°C under gassing with 100% O₂. The standard incubation buffer contained 10 mM sodium HEPES, 128 mM NaCl, 4.7 mM KCl, 0.58 mM MgCl₂, 0.55 mM Na₂HPO₄, 1.27 mM CaCl₂, 11.1 mM glucose, 20 mM glutamine, 1X amino acid solution (catalog no. 03-150-50; Sefar America Inc., Depew, NY) to obtain pancreatic acini. The acini were allowed to settle down on the bottom of a 15-ml Falcon tube, and the digestion buffer was removed. Acini from the pancreas of one mouse were resuspended in 5 ml of standard incubation buffer and preincubated for 15 min at 37°C under gassing with 100% O₂. The standard incubation buffer contained 10 mM sodium HEPES, 128 mM NaCl, 4.7 mM KCl, 0.58 mM MgCl₂, 0.55 mM Na₂HPO₄, 1.27 mM CaCl₂, 11.1 mM glucose, 20 mM glutamine, 1X amino acid solution, 0.2% bovine serum albumin, and 0.1% soybean trypsin inhibitor. Before use, the incubation buffer was gassed with 100% O₂ for 20 min and adjusted to pH 7.4. Incubated pancreata were then incubated in 5 ml of digestion buffer for 10 min at 37°C, followed by a 40-min incubation at 37°C using 5 ml of fresh digestion buffer. Throughout all incubations, samples were gassed with 95% O₂ supplemented with 5% CO₂. The digested pancreata were dispersed mechanically by repeatedly pipetting up and down using a 10-ml Nalgene pipette and then filtered through a nylon mesh (catalog no. 03-150-50; Sefar America Inc., Depew, NY) to obtain pancreatic acini. The acini were allowed to settle down on the bottom of a 15-ml Falcon tube, and the digestion buffer was removed.

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agent consists of water-insoluble cross-linked starch colored with a blue dye (Cséka et al., 1969). Samples were then processed for amylase measurements as described in the next paragraph. Amylase release was calculated as the percentage of amylase activity in the acini at the beginning of the incubation that was released into the extracellular medium during the 30-min incubation.

**Serum Amylase Measurements.** Blood was collected via retro-orbital puncture in EDTA-containing Eppendorf tubes. Serum was obtained by centrifugation (10 min at 4°C) at 1200g using an Eppendorf benchtop centrifuge. Serum (50 µl) was mixed with 4 ml of distilled water and prerincubated for 5 min at 37°C. After the addition of one Phadebas amylase test tablet (Pfizer, Inc.) to each tube, samples were incubated for 15 min at 37°C in a shaking water bath (200 cycles/min). The reaction was stopped by adding 1 ml of 0.5 M NaOH. Samples were vortexed immediately and centrifuged for 5 min at 1500g. After the centrifugation step, the absorbance of the supernatant was measured at 620 nm using a Pfizer spectrophotometer. Amylase activities were expressed in units per liter using the standard curve provided by the manufacturer (Pfizer, Inc.).

**Quantitative Real-Time PCR (TaqMan) Analysis of cDNA Prepared From Pancreatic Acini of WT and mACHR Mutant Mice.** Pancreatic acini were prepared from three age-matched WT, M₁ receptor KO, and M₃ receptor KO mice and immediately snap frozen in liquid nitrogen. Total RNA was obtained from the individual tissue samples by homogenization in TRIzol (Invitrogen) and purified as described previously (Gautam et al., 2004). Acinar RNA (1 µg) was reverse-transcribed in a final volume of 100 µl by using the high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA), following the manufacturer’s instructions.

Real-time PCR (TaqMan) was carried out on a 7900 HT sequence detection system (Applied Biosystems) using a 96-well format (final volume of reaction mixtures 50 µl). 18S rRNA (TaqMan RNA control reagents, VIC probe; Applied Biosystems) was used as an internal standard for normalization of mACHR mRNA in each reaction. The sequences of the mouse M₁–M₅ receptor-specific primers and TaqMan probes have been reported previously (Gautam et al., 2004).

TaqMan reaction mixtures contained 5 µl from the cDNA reaction (corresponding to 50 ng of total RNA), 1× TaqMan Universal master mix (Applied Biosystems) and all primers and probes at a final concentration of 200 nM. The cycling conditions were as follows: 10 min at 95°C for activation of AmpliTaq gold DNA polymerase, followed by 40 cycles of 15 s at 95°C for denaturation and 1 min at 60°C for annealing and amplification. All reverse transcriptase (RT) reactions and TaqMan assays were carried out in duplicate. RNA samples that had not been treated with RT were included as negative controls, confirming the absence of contaminating genomic DNA (data not shown). To compare relative mACHR expression levels (Ct values (Ct[mACHR] − Ct[control] = 18S rRNA)) (Ct, threshold cycle) were determined for all samples (Livak and Schmittgen, 2001).

**Immunoprecipitation Assays.** M₁ and M₃ receptor-specific rabbit polyclonal antisera were raised against nonconserved regions of the third cytoplasmic loops of the mouse M₁ and M₃ receptor proteins, respectively (Miyakawa et al., 2001; Yamada et al., 2001; Fisahn et al., 2002). The mACHR subtype selectivity of these antisera has been demonstrated previously (Miyakawa et al., 2001; Yamada et al., 2001; Fisahn et al., 2002).

Dispersed pancreatic acini were prepared as described in the previous paragraph. Pancreatic acini from one mouse were resuspended and homogenized for 20 s using a Brinkmann homogenizer in 5 ml of buffer A containing 25 mM sodium phosphate, 5 mM MgCl₂, pH 7.4, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor cocktail (catalog no. P2714; Sigma-Aldrich). Subsequently, aliquots of membrane homogenates were incubated in 1.5-ml Eppendorf tubes with 2 nM [³H]QNB in 1 ml of buffer A at room temperature for 1 h with end-over-end rotation. To remove unbound [³H]QNB, samples were then centrifuged at 8000g for 10 min at 4°C. The supernatants were discarded, and the pellets were resuspended in 1 ml of buffer A. This centrifugation/washing step was repeated twice. After the final washing step, pellets were resuspended in 1 ml of buffer A containing 1% digitonin. For precleaving, 20 µl of protein A-Sepharose beads was added, and samples were incubated at 4°C for 30 min with end-over-end rotation. Samples were then centrifuged at maximum speed in a refrigerated Eppendorf 5417R microcentrifuge for 2 min at 4°C, and supernatants (0.95 ml) were transferred to new Eppendorf tubes. The supernatants were then incubated with 30 µl of protein A-Sepharose beads at 4°C for 12 to 16 h with end-over-end rotation, with or without M₁ or M₃ mACHR-specific rabbit antisera (Miyakawa et al., 2001; Yamada et al., 2001; Fisahn et al., 2002). To wash out nonspecifically bound [³H]QNB, samples were centrifuged at maximum speed in a refrigerated Eppendorf 5417R microcentrifuge for 2 min at 4°C, and pellets were resuspended in 1 ml of buffer A containing 0.1% of digitonin. This centrifugation/washing step was repeated twice. After the final washing step, pellets were resuspended in 300 µl of Hydrofluor (National Diagnostics, Manville, NJ), and bound radioactivity was counted using a liquid scintillation counter.

**Morphological Studies.** Pancreata were harvested from WT and mACHR mutant mice. Tissues were fixed in 10% neutral buffered formalin, processed into paraffin blocks, sectioned at 6 µm, and stained with hematoxylin and eosin. Stained sections were examined by light microscopy (Olympus BX41). Photographs were taken with an Olympus DP12 digital camera, using the 20× lens.

**Statistics.** Statistical significance between two or more groups was determined by Student’s t test or one-way analysis of variance using post hoc t tests (Bonferroni’s method).

### Results

**Carbachol-Induced Amylase Secretion from Dispersed Pancreatic Acini Prepared from WT and M₁ and M₃ Receptor Single KO Mice.** To examine the role of the M₁ and M₃ mACHRs in muscarinic agonist-induced pancreatic amylase release, we initially prepared dispersed pancreatic acini from WT and M₁ and M₃ receptor single KO mice. The weight of the pancreata (percentage of total body weight) of the mACHR mutant mice did not differ significantly from that of the WT control mice (pancreas wet weight in milligrams per gram of body weight; WT, 8.8 ± 0.3; M₁ KO, 8.8 ± 1.0; M₃ KO, 8.1 ± 0.7; n = 5). We then studied the ability of the muscarinic agonist carbachol to stimulate amylase secretion using acinar preparations from the WT and the two mACHR mutant mouse strains. As shown in Fig. 1, the lack of functional M₁ or M₃ receptors had little effect on basal amylase release. In acinar preparations from WT mice, carbachol treatment led to concentration-dependent increases in amylase secretion (Fig. 1). At the highest carbachol concentrations used (≥10⁻⁵ M), the magnitude of these responses started to decline, consistent with previous observations (Gardner and Jensen, 1993). Strikingly, maximum secretory responses to carbachol (Eₘₕₜ × value) were significantly decreased (by ~40%, compared with the corresponding WT Eₘₜ × value) in preparations from both M₁ and M₃ receptor single KO mice (Fig. 1). In addition, carbachol showed an ~10-fold reduction in its potency to stimulate amylase release in acinar preparations from M₃ receptor single KO mice, compared with the corresponding WT preparations (WT, EC₅₀ ~3 × 10⁻⁷ M; M₁ KO, EC₅₀ ~3 × 10⁻⁶ M). In contrast, carbachol retained the ability to stimulate amylase secretion in acinar preparations from M₁ receptor single KO mice with high potency (EC₅₀ ~3 × 10⁻⁷ M).

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Fig. 1. Carbachol-induced increases in amylase secretion from dispersed pancreatic acini prepared from WT and M1 and M3 receptor single KO mice. Dispersed pancreatic acini were incubated with the indicated concentrations of carbachol for 30 min at 37°C. Results are expressed as the percentage of total cellular amylase activity before the incubation that was released into the extracellular medium during the incubation. Data are given as means ± S.E.M. of three independent experiments (sets of mice), each carried out in duplicate. *, P < 0.05; **, P < 0.01, compared with the corresponding WT values.

Analysis of mAChR Gene Expression in Mouse Pancreatic Acini by Quantitative Real-Time PCR (TaqMan). We next wanted to exclude the possibility that the magnitude of the secretion responses observed with acinar preparations from M1 and M3 receptor single KO mice was affected by altered expression levels of the remaining mAChR subtypes (e.g., overexpression of M1 receptors in M3 receptor KO mice, or vice versa, overexpression of M3 receptors in M1 receptor KO mice). To address this issue, we used quantitative real-time PCR (TaqMan) to determine the relative expression levels of the M1–M4 receptor transcripts in mouse pancreatic acini from WT and M1 and M3 receptor single KO mice. Relative mAChR expression levels were compared between WT samples and samples from M1 or M3 receptor single KO mice by comparing ΔCt values (Ct[mAChR] - Ct[internal control = 18S rRNA]) (Livak and Schmittgen, 2001).

Quantitative real-time PCR analysis of cDNA prepared from mouse pancreatic acini indicated that M1 receptor transcripts were present at similar levels in WT and M3 receptor KO mice (Fig. 2). Similarly, M1 receptor mRNA was expressed at similar levels in WT and M1 receptor KO mice (Fig. 2). TaqMan analysis also demonstrated the presence of low levels (ΔCt values >25) of M2 and M4 receptor transcripts in mouse pancreatic acini (Fig. 2), but it failed to demonstrate the presence of M5 receptor mRNA (data not shown). The absence of functional M1 or M3 receptors had no significant effect on M2 and M4 receptor mRNA expression levels (Fig. 2). These data indicate that inactivation of the M1 or M3 mAChR genes does not result in significantly altered mRNA levels of the remaining mAChR subtypes expressed in mouse pancreatic acini.

Expression of M1 and M3 mAChR Protein in Mouse Pancreatic Acini Studied by an Immunoprecipitation Strategy. As described above, carbachol-induced pancreatic amylase secretion was impaired more severely in acinar preparations from M3 than from M1 receptor KO mice. To study whether these functional differences were associated with differences in receptor densities, we studied the expression of M1 and M3 receptor protein in mouse pancreatic acini by using receptor subtype-specific antisera (Miyakawa et al., 2001; Yamada et al., 2001; Fisahn et al., 2002).

Specifically, mAChRs present in mouse acinar membrane preparations were labeled with a receptor-saturating concentration (2 nM) of the nonsubtype-selective muscarinic antagonist [3H]QNB, solubilized with 1% digitonin, and then immunoprecipitated by using M1 or M3 receptor-specific antisera (Miyakawa et al., 2001; Yamada et al., 2001; Fisahn et al., 2002). As described previously (Miyakawa et al., 2001; Yamada et al., 2001; Fisahn et al., 2002), these antisera were raised in rabbits against nonconserved regions of the third cytoplasmic loops of the mouse M1 and M3 receptor proteins. These immunoprecipitation studies showed that mouse pancreatic acini expressed ~40 to 50% more M3 than M1 receptors (Fig. 3). The M1 and M3 receptor-selective antisera did not precipitate significant amounts of radioactivity in M1 and M3 KO mice.

Fig. 2. Quantitative RT-PCR analysis (TaqMan) of mAChR subtype expression in pancreatic acini from WT and mAChR mutant mice. Quantitative RT-PCR analysis (TaqMan) was used to determine the relative levels of M1–M4 mAChR transcripts in pancreatic acinar tissue from WT and M1 and M3 receptor single KO mice. No specific signal was obtained by the use of M1 receptor-specific primers and probes, indicative of the absence of measurable levels of M1 receptor mRNA. The sequences of the M1–M4 mAChR-selective TaqMan primers and probes have been reported previously (Gautam et al., 2004). Relative mAChR mRNA levels were expressed as ΔCt values (Ct[mAChR] - Ct[internal control = 18S rRNA]) (Livak and Schmittgen, 2001). Note that mAChR expression levels are similar in the presence or absence of functional M1 or M3 receptors. Data are presented as means ± S.D. from three different experiments (sets of mice), each carried out in duplicate.
M3 receptor KO mice, respectively, consistent with the receptor subtype-specificity of these antisera (Fig. 3).

**Carbachol-Induced Pancreatic Amylase Secretion Is Abolished in M1/M3 Receptor Double KO Mice.** To examine whether the activation of M1 and M3 mAChRs fully accounts for carbachol-induced pancreatic amylase release, we next tested the ability of carbachol to stimulate amylase secretion from dispersed pancreatic acini prepared from M1/M3 receptor double KO and their corresponding WT control mice. These studies showed that carbachol-mediated pancreatic amylase secretion was abolished in acinar preparations from M1/M3 receptor double KO mice (Fig. 4A).

The weight of the pancreata (percentage of total body weight) of the M1/M3 receptor double KO mice did not differ significantly from that of the corresponding WT control mice (pancreas wet weight in milligrams per gram of body weight: WT, 8.6 ± 0.3; M1/M3 KO, 8.1 ± 0.7; n = 5). Moreover, total pancreatic amylase content was not significantly different between WT and M1/M3 receptor double KO mice (units per milligram of pancreatic wet weight: WT, 11.2 ± 0.4; M1/M3 KO, 15.5 ± 2.0; n = 6).

In contrast to the inability of carbachol to stimulate pancreatic amylase secretion in M1/M3 receptor double KO mice, the secretory response to bombesin, which interacts with receptors that are coupled to G proteins of the Gq family, similar to the M1 and M3 mAChRs, remained unaffected by the simultaneous lack of functional M1 and M3 receptors (Fig. 4B). This observation suggested that the simultaneous disruption of the M1 and M3 mAChR genes did not interfere with the integrity of the downstream signal transduction cascades critically involved in pancreatic amylase secretion.

In addition, light microscopic studies showed that the morphology of pancreatic exocrine tissue from M1/M3 receptor double KO mice did not differ from that of their corresponding WT controls (Fig. 5, A and D). Similar results were obtained with pancreatic sections from M1 and M3 receptor single KO mice (Fig. 5, A and C).

**Serum Amylase Levels Are Significantly Reduced in M1/M3 Receptor Double KO Mice.** Considerable evidence suggests that pancreatic amylase can enter the circulation via a direct “leak” from pancreatic acini or ducts into the

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**Fig. 3.** Expression of M1 and M3 mAChR protein in mouse pancreatic acini studied by using an immunoprecipitation strategy. Membranes prepared from pancreatic acini from WT and M1 and M3 receptor KO mice were incubated with 2 nM nonselective muscarinic antagonist [3H]QNB. [3H]QNB-labeled mAChRs were solubilized and immunoprecipitated with M1 or M3 receptor-selective antisera, as described under Materials and Methods. The numbers of immunoprecipitated receptors are expressed as femtomoles of [3H]QNB binding sites per mg membrane protein. Data are given as means ± S.E.M. (five mice per group).

**Fig. 4.** Effects of carbachol and bombesin on amylase secretion from dispersed pancreatic acini prepared from WT and M1/M3 receptor double KO mice. A, carbachol-induced amylase secretion is abolished in acinar preparations from M1/M3 receptor double KO mice. B, bombesin-induced amylase secretion remains unaffected by the lack of functional M1 and M3 mAChRs. Dispersed pancreatic acini were incubated with increasing concentrations of carbachol or bombesin for 30 min at 37°C. Results are expressed as the percentage of total cellular amylase activity before the incubation that was released into the extracellular medium during the incubation. Data are given as means ± S.E.M. of three independent experiments (sets of mice), each carried out in duplicate.

**Fig. 5.** Histological profile of pancreatic exocrine tissue from WT and mAChR mutant mice. A, WT. B, M1 receptor single KO. C, M3 receptor single KO. D, M1/M3 receptor double KO mice. Sections were stained with hematoxylin and eosin and observed by light microscopy. Bar, 20 μm.
blood (Pieper-Bigelow et al., 1990). To examine whether the lack of mAChR-mediated stimulation of amylase secretion displayed by the $M_1/M_3$ receptor double KO mice was associated with changes in serum amylase levels, we determined serum amylase levels in $M_1$ and $M_3$ receptor single and $M_1/M_3$ receptor double KO mice. We found that serum amylase levels were not significantly different in $M_1$ and $M_3$ receptor single KO mice, compared with the corresponding levels displayed by WT mice (Fig. 6). In contrast, serum amylase levels were significantly reduced (by $\sim 50\%$) in $M_1/M_3$ receptor double KO mice (Fig. 6).

**Discussion**

The extrinsic parasympathetic innervation of the pancreas is known to play a key role in inducing the secretion of digestive enzymes from the exocrine pancreas (Holst, 1993). In the present study, we examined the potential functional roles of pancreatic $M_1$ and $M_3$ mAChRs in mediating this activity. Specifically, we assessed carbachol-induced amylase secretion in vitro, using dispersed pancreatic acini prepared from $M_1$ and $M_3$ receptor single KO and $M_1/M_3$ receptor double KO mice.

This analysis showed that maximum secretory response to carbachol ($E_{\text{max}}$ values) were decreased by $\sim 40\%$ in both $M_1$ and $M_3$ receptor single KO mice (Fig. 1). In addition, carbachol was about 10-fold less potent in acinar preparations from $M_3$ receptor single KO mice ($EC_{50} \approx 3 \times 10^{-8} \text{M}$). On the other hand, carbachol showed similar potencies in preparations from $M_1$ receptor KO and WT mice ($EC_{50} \approx 3 \times 10^{-7} \text{M}$). Strikingly, carbachol-induced amylase secretion was abolished in pancreatic acinar preparations from $M_1/M_3$ receptor double KO mice (Fig. 4A). In contrast, bombesin, another potent stimulant of pancreatic exocrine secretion, fully retained its ability to stimulate amylase secretion in the absence of both $M_1$ and $M_3$ receptors (Fig. 4B). Like the $M_1$ and $M_3$ mAChRs, bombesin is predicted to stimulate exocrine pancreas secretion by activation of a receptor that is linked to G proteins of the $G_\alpha$ family (Hampton et al., 1998). These G proteins are known to mediate the activation of different isoforms of phospholipase C$\beta$, followed, among other responses, by the generation of the second messengers diacylglycerol and inositol 1,4,5-triphosphate, the increase in free intracellular calcium levels, and the activation of a series of protein kinases. These events are predicted to eventually trigger enzyme secretion in pancreatic acinar cells, although the precise sequence of events underlying this activity remains to be elucidated (Williams and Yule, 1993). Regardless, that pancreatic acini from $M_1/M_3$ receptor double KO mice remained fully responsive to the secretagogue bombesin indicates that the functional deficits displayed by these acini were carbachol-specific and not caused by disruption of downstream signaling events.

Immunoprecipitation studies with $M_1$ and $M_3$ receptor-selective antisera showed that mouse pancreatic acinar cells express significantly more $M_3$ than $M_1$ receptors (Fig. 3), providing a possible explanation for the observation that the lack of $M_3$ receptors led to a more pronounced inhibition in carbachol-induced amylase secretion (reduction in $E_{\text{max}}$ and carbachol potency) than the lack of $M_1$ receptors. Quantitative real-time RT-PCR experiments (TaqMan) demonstrated the presence of $M_1$-$M_3$ receptor mRNA in mouse pancreatic acinar preparations but failed to detect significant levels of $M_1$ receptor mRNA (Fig. 2). Importantly, genetic inactivation of the $M_1$ or $M_3$ mAChR genes had no significant effect on the mRNA levels of the remaining mAChR subtypes in mouse pancreatic acini (Fig. 2). It is therefore unlikely that the shape or position of the carbachol concentration-response curves observed with the $M_1$ and $M_3$ receptor single KO preparations was significantly affected by the up- or down-regulation of other mAChR subtypes.

Radioligand binding studies with transiently transfected COS-7 cells showed that carbachol displayed similar binding affinities for the mouse $M_1$ and $M_3$ mAChRs ($K_i \approx 6 \mu\text{M}$; J.-W. Gan and J. Wess, unpublished data), as has been observed with the human $M_1$ and $M_3$ mAChRs (Peralta et al., 1987). This finding excludes the possibility that the $\sim 10$-fold rightward shift of the carbachol concentration-response curve observed with acinar preparations from $M_3$ receptor KO is caused by differences in carbachol binding affinities at the two receptor subtypes. One possible explanation for the reduction in carbachol potency observed with acini from $M_3$ receptor KO mice is the fact that $M_1$ receptors are expressed at lower levels than $M_3$ receptors (Fig. 3). In addition, other factors, such as differences in subcellular distribution patterns and G protein coupling profiles of the two receptor subtypes (Burford et al., 1995) may also contribute to this phenomenon.

It has been suggested, primarily on the basis of pharmacological studies using muscarinic agonists of limited receptor subtype selectivity, that muscarinic stimulation of exocrine pancreas function is virtually exclusively controlled by the $M_3$ receptor subtype (Louie and Owyang, 1986; Korc et al., 1987; Iwatsuki et al., 1988; van Zwan et al., 1990; Kato et al., 1992; Love et al., 1999). However, in the present study we provide unambiguous evidence that cholinergic activation of pancreatic digestive enzyme (amylase) secretion is mediated by a mixture of $M_1$ and $M_3$ mAChRs, at least in the mouse.

![Fig. 6. Serum amylase levels of WT and mAChR mutant mouse. Serum amylase levels were determined in $M_1$ and $M_3$ receptor single and $M_1/M_3$ receptor double KO mice, as described under Materials and Methods. Data are given as means ± S.E.M. (6–15 mice per group). **, $P < 0.01$, compared with the corresponding WT value.](https://example.com/f6.png)
Several years ago, Schmid et al. (1998) reported that the M1 receptor-prefering antagonist telenzepine (a pirenzepine analog) was about 1000 times more potent than 4-DAMP in inhibiting carbachol-induced amylase secretion in rat pancreatic acini. However, 4-DAMP (Dörje et al., 1991; Caulfield and Birdsall, 1998) and telenzepine (Schudt et al., 1989; Lazareno et al., 1990; Karton et al., 1991) are known to bind to M1 receptors with similar affinities \((K_i \sim 1–3 \text{ nM})\), and even high concentrations of the classical M3 receptor-prefering antagonist pirenzepine had no effect on carbachol-induced amylase secretion (Schmid et al., 1998). The authors therefore suggested that a “distinct form” of mAChR may be present on pancreatic acinar cells. However, our data indicate that a mixture of M1 and M3 receptors fully accounts for the carbachol-induced amylase secretion from mouse pancreatic acini.

A series of in vivo studies in human and dogs also indicated that telenzepine can inhibit basal and stimulated pancreatic exocrine output with high potency (for a recent review of these studies, see Niebergall-Roth and Singer, 2003). Based on these findings it has been proposed that M1 receptors play an important role in regulating pancreatic exocrine secretion (Niebergall-Roth and Singer, 2003). However, radioligand binding studies indicate that telenzepine not only binds to M1 receptors with high affinity \((K_i \sim 1 \text{ nM})\) but also shows relatively high affinity for M3 receptors \((K_i \sim 4–8 \text{ nM})\;\text{Schudt et al., 1989; Lazareno et al., 1990; Karton et al., 1991}\). Moreover, in vivo studies, pharmacokinetic factors greatly complicate the proper interpretation of results obtained with ligands of limited receptor subtype specificity. Because of these reasons, it is difficult to assess the relative contribution of M1 and M3 receptors to pancreatic exocrine output by the use of telenzepine in vivo. In the present study, we were able to circumvent these difficulties by the use of mAChR KO mice.

Besides ACh, several gastrointestinal peptides, including cholecystokinin (CCK), bombesin-related peptides, vasoactive intestinal peptide, and secretin, can also act on pancreatic acinar cells to stimulate digestive enzyme secretion (Gardner and Jensen, 1993; Chey and Chang, 2001). Like the mAChRs, the receptors mediating the secretory activity of these peptides belong to the superfamily of G protein-coupled receptors (Gardner and Jensen, 1993). Using gene targeting technology in mice, recent studies have begun to reveal the identity of the receptor subtypes involved in the secretory actions of these peptides. These studies have shown, for example, that the ability of CCK and bombesin to stimulate amylase release from the exocrine pancreas is mediated by CCK\(_A\) (Takiguchi et al., 2002) and gastrin-releasing peptide receptors (Hampton et al., 1998), respectively, at least in the mouse.

Interestingly, we found that serum amylase levels were reduced by \(\sim 50\%\) in M1/M3 receptor double KO mice (Fig. 6). Several studies suggest that the exocrine pancreas and the salivary glands are the major sources of serum amylase (Amerongen et al., 1980; Pieper-Bigelow et al., 1990). Pancreatic amylase is predicted to enter the circulation via a direct leak from pancreatic acini or ducts into the blood (Pieper-Bigelow et al., 1990). Since pancreata of M1/M3 receptor double KO mice contained similar amounts of amylase as pancreata of WT mice, it is likely that the lack of ACh-stimulated pancreatic amylase release is responsible, at least partially, for the reduction in serum amylase levels displayed by the M1/M3 receptor double KO mice. Besides the exocrine pancreas, salivary glands, particularly the parotid gland, also release substantial amounts of amylase into the circulation (Amerongen et al., 1980). We (Gautam et al., 2004) and Nakamura et al. (2004) recently demonstrated that muscarinic agonist (pilocarpine)-induced increases in salivary flow are abolished in M1/M3 receptor double KO mice. It is therefore possible that the lack of ACh-stimulated salivation also contributes to the reduction in serum amylase levels observed with the M1/M3 receptor double KO mice.

mAChRs are not only expressed by cells of the exocrine pancreas but also by islet cells constituting the endothrine pancreas (Ahren, 2000). In contrast to the findings reported here, recent studies with isolated islets showed that disruption of the M3 receptor gene was sufficient to abolish muscarinic agonist-induced insulin secretion (Duttaroy et al., 2004; Zawalich et al., 2004) and glucagon (Duttaroy et al., 2004) release. Analysis of pancreatic islets from M1 receptor KO mice showed that muscarinic agonist-induced insulin release remained unaltered in the absence of M1 receptors (D. Mears, C. Zimliki, and J. Wess, unpublished data). As already mentioned above, in vivo studies with mAChR mutant mice demonstrated that cholinergic stimulation of salivary flow is mediated by a mixture of M1 and M3 mAChRs (Gautam et al., 2004; Nakamura et al., 2004). Future studies will show whether M1 receptors, together with M3 receptors, generally participate in the function of exocrine glands.

In conclusion, our results indicate that cholinergic stimulation of pancreatic amylase release is mediated by a mixture of M1 and M3 mAChRs and that other mAChR subtypes do not contribute to this activity. These findings clarify the long-standing question regarding the molecular nature of the mAChRs mediating the secretion of digestive enzymes from the exocrine pancreas. Given the vital importance of exocrine pancreatic output, our findings should be of considerable clinical interest.

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