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

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Running title: Muscarinic receptor-mediated amylase secretion

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ABBREVIATIONS:

ACh, acetylcholine; C_t, threshold cycle; CCK, cholecystokinin; KO, knockout; mAChR, muscarinic acetylcholine receptor; [^{3}H]QNB, [^{3}H]Quinuclidinyl benzilate; RT, reverse transcriptase; WT, wild-type.

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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 M_1 and M_3 mAChRs in this activity, we used M_1 and M_3 receptor single knockout (KO) and M_1/M_3 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 RT-PCR studies using RNA prepared from mouse pancreatic acini showed that deletion of the M_1 or M_3 mAChR genes did not lead to significantly altered mRNA levels of the remaining mAChR subtypes. Moreover, immunoprecipitation studies with M_1 and M_3 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 M_1 and M_3 receptor single KO mice and completely abolished in acinar preparations from M_1/M_3 receptor double KO mice. However, another pancreatic secretagogue, bombesin, retained its ability to fully stimulate amylase secretion in acinar preparations from M_1/M_3 receptor double KO mice. Taken together these studies support the concept that cholinergic stimulation of pancreatic amylase secretion is mediated by a mixture of M_1 and M_3 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.

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Introduction

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). Following 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; Posssani 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 (M_1-M_5; Wess, 1996; Caulfield and Birdsall, 1998). The M_1, M_3, and M_5 receptors are selectively coupled to G proteins of the G_q family which mediate the breakdown of phosphatidyl inositol lipids, whereas the M_2 and M_4 receptors are preferentially linked to G proteins of the G_i class which, at a biochemical level, mediate the inhibition of adenylyl cyclase (Wess, 1996; Caulfield and Birdsall, 1998).

In the past, several groups have reported that ACh (muscarinic agonist)-induced stimulation of exocrine pancreas secretion is mediated by the M_3 receptor subtype (Louie and Owyang, 1986; Korc et al., 1987; Iwatsuki et al., 1989; van Zwam et al., 1990; Kato

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et al., 1992; Love et al., 1999). This conclusion was based on radioligand binding and functional studies employing several subtype-preferring muscarinic antagonists, such as pirenzepine (preferentially binds to M_1 receptors), AF-DX 116 (preferentially binds to M_2 and M_4 receptors), and 4-DAMP (has ~10 higher affinity for M_1, M_3, M_4, and M_5 receptors than for M_2 receptors) (Dörje et al., 1991; Wess, 1996; Caulfield and Birdsall, 1998). However, the subtype 'selectivity' of these antagonists, like that of most other subtype-preferring muscarinic antagonists currently used as pharmacological tools, is rather limited (Wess, 1996; Caulfield and Birdsall, 1998). The possibility therefore exists that other mAChR subtypes, which may be less abundant than the M_3 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 M_1, M_2, M_3, M_4, or M_5 mAChRs (for a 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 do not only express M_3 but also M_1 mAChR mRNA (Schmid et al., 1998; Turner et al., 2001). To examine whether both acinar M_1 and M_3 receptors play a role in cholinergic stimulation of exocrine pancreas secretion, we used M_1 (Miyakawa et al., 2001; Fisahn et al., 2002) and

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M_3 receptor single KO mice (Yamada et al., 2001) and recently generated M_1/M_3 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 (Yamada et al., 2001; Miyakawa 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 M_1 and M_3 receptor single KO mice and completely abolished in acinar preparations from M_1/M_3 receptor double KO mice. These data indicate that mAChR-mediated stimulation of amylase secretion from the exocrine pancreas is mediated by a mixture of M_1 and M_3 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.

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Materials and Methods

Materials. Unless noted otherwise, all chemicals, including carbachol (carbamoyl chloride) and bombesin, were purchased from Sigma. Collagenase was obtained from Crescent Chemicals (cat. # 1745N). [^{3}H]Quinuclidinyl benzilate ([^{3}H]QNB; specific activity: 42 Ci/mmol) was purchased from PerkinElmer. Protein A Sepharose^{TM} CL-4B beads were obtained from Amersham Biosciences.

Muscarinic Receptor Mutant Mice. The generation of homozygous M_1 receptor single KO mice (Miyakawa et al., 2001; Fisahn et al., 2002), homozygous M_3 receptor single KO mice (Yamada et al., 2001), and homozygous M_1/M_3 receptor double KO mice (Gautam et al., 2004) has been described previously. All mutant mice and the corresponding WT strains had the same mixed genetic background (129SvEv [50%] x CF1 [50%]).

All experiments were performed during the light cycle using adult male mice that were 3-6 months old. In all experiments, KO mice and age-matched WT mice of the same genetic background were used in parallel. All animal studies were conducted according to the NIDDK guidelines for standard animal care and usage.

Preparation of Isolated Mouse Pancreatic Acini. Dispersed mouse pancreatic acini were prepared as described previously (Matozaki et al., 1990), with some modifications. In brief, pancreata were excised carefully from WT or mAChR mutant mice and injected with ~5 ml of digestion buffer. The composition of the digestion buffer was as follows:

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collagenase (10 U/ml), 110 mM NaCl, 4.7 mM KCl, 1.16 mM $MgCl_2$, 0.55 mM Na_2HPO_4, 0.5 mM $CaCl_2$, 22.2 mM glucose, 20 mM glutamine, 1 x amino acid solution (Gibco, cat. # 0552), 0.025% bovine serum albumin, and 0.1% soybean trypsin inhibitor. Prior to injection, the digestion buffer was gassed with 95% O_2 and 5% CO_2 for 20 min and adjusted to pH 7.4. Injected pancreata were then incubated in 5 ml of digestion buffer for 10 min at 37 ^{0}C, followed by a 40 min incubation at 37 ^{0}C using 5 ml of fresh digestion buffer. Throughout all incubations, samples were gassed with 95% O_2 supplemented with 5% CO_2. 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 (Nitex, cat. # 03-150-50) 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 ^{0}C under gassing with 100% O_2. The standard incubation buffer contained 10 mM sodium HEPES, 128 mM NaCl, 4.7 mM KCl, 0.58 mM $MgCl_2$, 0.55 mM Na_2HPO_4, 1.27 mM $CaCl_2$, 11.1 mM glucose, 20 mM glutamine, 1 x amino acid solution, 0.2% bovine serum albumin, and 0.1% soybean trypsin inhibitor. Prior to use, the incubation buffer was gassed with 100% O_2 for 20 min and adjusted to pH 7.4.

Amylase 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 (see previous paragraph), and 1 ml aliquots were

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incubated in duplicate with increasing concentrations of carbachol or bombesin for 30 min at 37 ^{0}C in a shaking water bath (60 cycles/min). In parallel, 1 ml aliquots (duplicates) of the cell suspension were transferred to 1.5 ml Eppendorf tubes, sonicated, and the total amount of amylase activity contained in these samples was determined by using the Phadebas® reagent (Pharmacia Diagnostics; see below), essentially as described by Williams et al. (1978). Throughout all incubations with carbachol or bombesin, samples were gassed with 100% O_2. After the 30 min incubation period, samples were transferred to 1.5 ml Eppendorf tubes and centrifuged for 2-3 sec at maximum speed in an Eppendorf bench top centrifuge. Aliquots of the supernatants (10 µl) were then mixed with 1 ml of amylase reaction buffer. To prepare the amylase reaction buffer, 1 tablet of Phadebas® reagent was dissolved in 14 ml of an aqueous solution containing 50 mM NaCl, 20 mM NaH_2PO_4, and 0.02% NaN_3 (sodium azide). The Phadebas® reagent consists of water-insoluble cross-linked starch colored with a blue dye (Ceska 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 ^{0}C) at 1,200 x g using an Eppendorf bench top centrifuge. 50 µl of serum were mixed with 4 ml of distilled water and preincubated for 5 min at 37 ^{0}C. After the addition of one Phadebas® amylase test tablet (Pharmacia Diagnostics) to each tube, samples were

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incubated for 15 min at 37 ^{0}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 1,500 x g. After the centrifugation step, the absorbance of the supernatant was measured at 620 nm using a Pharmacia Biotech spectrophotometer. Amylase activities were expressed in U/l using the standard curve provided by the manufacturer (Pharmacia Diagnostics).

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, M_1 receptor KO, and M_3 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), 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 M_1-M_5 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 x TaqMan Universal Master Mix (Applied Biosystems) and all

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primers and probes at a final concentration of 200 nM. The cycling conditions were as follows: 10 min at 95 ^{o}C for activation of AmpliTaq Gold DNA polymerase, followed by 40 cycles of 15 sec at 95 ^{o}C for denaturation and 1 min at 60 ^{o}C for annealing and amplification. All RT reactions and TaqMan assays were carried out in duplicate. RNA samples that had not been treated with reverse transcriptase (RT) were included as negative controls, confirming the absence of contaminating genomic DNA (data not shown). In order to compare relative mAChR expression levels, ΔC_t values (C_t[mAChR]-C_t[control=18S rRNA]) (C_t =threshold cycle) were determined for all samples (Livak and Schmittgen, 2001).

Immunoprecipitation Assays. M_1 and M_3 receptor-specific rabbit polyclonal antisera were raised against non-conserved regions of the third cytoplasmic loops of the mouse M_1 and M_3 receptor proteins, respectively (Yamada et al., 2001; Miyakawa et al., 2001; Fisahn et al., 2002). The mAChR subtype selectivity of these antisera has been demonstrated previously (Yamada et al., 2001; Miyakawa 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 sec using a Brinkmann homogenizer in 5 ml of buffer A containing 25 mM sodium phosphate, 5 mM $MgCl_2$ (pH 7.4), 1 mM phenylmethylsulphonyl fluoride, and 1 x protease inhibitor cocktail (Sigma, cat. # P2714). Subsequently, aliquots of membrane homogenates were incubated in 1.5 ml Eppendorf tubes with 2 nM [^{3}H]QNB in 1 ml of buffer A at room temperature for 1 hr with end-over-end rotation. To remove unbound [^{3}H]QNB, samples

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were then centrifuged at 8000 x g for 10 min at 4 ^{0}C. The supernatants were discarded and the pellets were resuspend 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 preclearing, 20 µl of protein A sepharose beads were added and samples were incubated at 4 ^{0}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 ^{0}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 ^{0}C for 12-16 hrs with end-over-end rotation, with or without M_1 or M_3 mAChR-specific rabbit antisera (Yamada et al., 2001; Miyakawa et al., 2001; Fisahn et al., 2002). To wash out non-specifically bound [^{3}H]QNB, samples were centrifuged at maximum speed in a refrigerated Eppendorf 5417R microcentrifuge for 2 min at 4 ^{0}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), 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 microns, 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 x lens.

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

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Results

Carbachol-induced Amylase Secretion From Dispersed Pancreatic Acini Prepared From WT and M_1 and M_3 Receptor Single KO Mice. To examine the role of the M_1 and M_3 mAChRs in muscarinic agonist-induced pancreatic amylase release, we initially prepared dispersed pancreatic acini from WT and M_1 and M_3 receptor single KO mice. The weight of the pancreata (% of total body weight) of the mAChR mutant mice did not differ significantly from that of the WT control mice (pancreas wet weight in mg/g body weight: WT, 8.8±0.3; M_1 KO, 8.8±1.0; M_3 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 M_1 or M_3 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 ($\geq 10^{-5}$ M), the magnitude of these responses started to decline, consistent with previous observations (Gardner and Jensen, 1993). Strikingly, maximum secretory responses to carbachol (E_{max} values) were significantly decreased (by ~40%, as compared to the corresponding WT E_{max} value) in preparations from both M_1 and M_3 receptor single KO mice (Fig. 1). In addition, carbachol showed a ~10 fold reduction in its potency to stimulate amylase release in acinar preparations from M_3 receptor single KO mice, as compared to the corresponding WT preparations (WT, EC_{50} ~3 x 10^{-7} M; M_3 KO, EC_{50} ~3 x 10^{-6} M). In contrast, carbachol retained the ability to stimulate amylase secretion in

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acinar preparations from M_1 receptor single KO mice with high potency (EC_{50} ~3 x 10^{-7} M).

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 M_1 and M_3 receptor single KO mice was affected by altered expression levels of the remaining mAChR subtypes (e.g. overexpression of M_1 receptors in M_3 receptor KO mice or, vice versa, overexpression of M_3 receptors in M_1 receptor KO mice). To address this issue, we used quantitative real-time PCR (TaqMan) to determine the relative expression levels of the M_1-M_5 receptor transcripts in mouse pancreatic acini from WT and M_1 and M_3 receptor single KO mice. Relative mAChR expression levels were compared between WT samples and samples from M_1 or M_3 receptor single KO mice by comparing ΔC_t values (C_t[mAChR]-C_t[internal control=18S rRNA]) (Livak and Schmittgen, 2001).

Quantitative real-time PCR analysis of cDNA prepared from mouse pancreatic acini indicated that M_1 receptor transcripts were present at similar levels in WT and M_3 receptor KO mice (Fig. 2). Similarly, M_3 receptor mRNA was expressed at similar levels in WT and M_1 receptor KO mice (Fig. 2). TaqMan analysis also demonstrated the presence of low levels (ΔC_t values >25) of M_2 and M_4 receptor transcripts in mouse pancreatic acini (Fig. 2), but failed to demonstrate the presence of M_5 receptor mRNA (data not shown). The absence of functional M_1 or M_3 receptors had no significant effect on M_2 and M_4 receptor mRNA expression levels (Fig. 2). These data indicate that inactivation of the M_1

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or M_3 mAChR genes does not result in significantly altered mRNA levels of the remaining mAChR subtypes expressed in mouse pancreatic acini.

Expression of M_1 and M_3 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 M_3 than from M_1 receptor KO mice. To study whether these functional differences were associated with differences in receptor densities, we studied the expression of M_1 and M_3 receptor protein in mouse pancreatic acini by using receptor subtype-specific antisera (Yamada et al., 2001; Miyakawa 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 non-subtype-selective muscarinic antagonist, [^{3}H]QNB, solubilized with 1% digitonin, and then immunoprecipitated by using M_1 or M_3 receptor-specific antisera (Yamada et al., 2001; Miyakawa et al., 2001; Fisahn et al., 2002). As described previously (Yamada et al., 2001; Miyakawa et al., 2001; Fisahn et al., 2002), these antisera were raised in rabbits against non-conserved regions of the third cytoplasmic loops of the mouse M_1 and M_3 receptor proteins. These immunoprecipitation studies showed that mouse pancreatic acini expressed ~40-50% more M_3 than M_1 receptors (Fig. 3). The M_1 and M_3 receptor-selective antisera did not precipitate significant amounts of radioactivity in M_1 and M_3 receptor KO mice, respectively, consistent with the receptor subtype-specificity of these antisera (Fig. 3).

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Carbachol-induced Pancreatic Amylase Secretion is Abolished in M_1/M_3 Receptor Double KO Mice. To examine whether the activation of M_1 and M_3 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 M_1/M_3 receptor double KO and their corresponding WT control mice. These studies showed that carbachol-mediated pancreatic amylase secretion was abolished in acinar preparations from M_1/M_3 receptor double KO mice (Fig. 4*A*).

The weight of the pancreata (% of total body weight) of the M_1/M_3 receptor double KO mice did not differ significantly from that of the corresponding WT control mice (pancreas wet weight in mg/g body weight: WT, 8.6±0.3; M_1/M_3 KO, 8.1±0.7; n=5). Moreover, total pancreatic amylase content was not significantly different between WT and M_1/M_3 receptor double KO mice (U/mg pancreatic wet weight: WT, 11.2±0.4; M_1/M_3 KO, 15.5±2.0; n=6).

In contrast to the inability of carbachol to stimulate pancreatic amylase secretion in M_1/M_3 receptor double KO mice, the secretory response to bombesin, which interacts with receptors that are coupled to G proteins of the G_q family, similar to the M_1 and M_3 mAChRs, remained unaffected by the simultaneous lack of functional M_1 and M_3 receptors (Fig. 4*B*). This observation suggested that the simultaneous disruption of the M_1 and M_3 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 M_1/M_3 receptor double KO mice did not differ from that of their

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corresponding WT controls (Fig. 5*A, D*). Similar results were obtained with pancreatic sections from M_1 and M_3 receptor single KO mice (Fig. 5*A-C*).

Serum Amylase Levels are Significantly Reduced in M_1/M_3 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 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, as compared to the corresponding levels displayed by WT mice (Fig. 6). In contrast, serum amylase levels were significantly reduced (by ~50%) in M_1/M_3 receptor double KO mice (Fig. 6).

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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_{max} values) were decreased by ~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} ~3 x 10^{-6} M). On the other hand, carbachol showed similar potencies in preparations from M_1 receptor KO and WT mice (EC_{50} ~3 x 10^{-7} M). Strikingly, carbachol-induced amylase secretion was completely abolished in pancreatic acinar preparations from M_1/M_3 receptor double KO mice (Fig. 4*A*). 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. 4*B*). 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_q family (Hampton et al., 1998). These G proteins are known to mediate the activation of different isoforms of phospholipase Cβ, followed, among other responses, by the generation of the second messengers diacylglycerol and inositol 1,4,5-trisphosphate, the increase in free intracellular calcium levels, and the activation of a series of protein kinases. These events

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are predicted to eventually trigger enzyme secretion in pancreatic acinar cells, although the precise sequence of events underling this activity remains to be elucidated (Williams and Yule, 1993). In any case, the fact 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_{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_4 receptor mRNA in mouse pancreatic acinar preparations but failed to detect significant levels of M_5 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 ~6 μM; J.-W. Gan and J. Wess, unpublished results), as has been observed with the human M_1 and M_3 mAChRs (Peralta et al., 1987). This finding excludes the possibility that the

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~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 antagonists 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., 1989; van Zwam 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.

Several years ago, Schmidt et al. (1998) reported that the M_1 receptor-preferring antagonist, telenzepine (a pirenzepine analog), was about 1,000 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 M_1 receptors with similar affinities (K_i ~1-3 nM), and even high concentrations of the classical M_1 receptor-preferring antagonist, pirenzepine, had no effect on carbachol-

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induced amylase secretion (Schmidt 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 M_1 and M_3 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 M_1 receptors play an important role in regulating pancreatic exocrine secretion (Niebergall-Roth and Singer, 2003). However, radioligand binding studies indicate that telenzepine does not only bind to M_1 receptors with high affinity (K_i ~1 nM) but also shows relatively high affinity for M_3 receptors (K_i ~ 4-8 nM; Schudt et al., 1989; Lazareno et al., 1990; Karton et al., 1991). Moreover, in *in vivo* studies, pharmacokinetic factors greatly complicate the proper interpretation of results obtained with ligands of limited receptor subtype selectivity. Because of these reasons, it is difficult to assess the relative contribution of M_1 and M_3 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 (VIP), 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

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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 (GRP) receptors (Hampton et al., 1998), respectively, at least in the mouse.

Interestingly, we found that serum amylase levels were reduced by ~50% in M_1/M_3 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 M_1/M_3 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 M_1/M_3 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 M_1/M_3 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 M_1/M_3 receptor double KO mice.

mAChRs are not only expressed by cells of the exocrine pancreas but also by islet cells constituting the endocrine pancreas (Ahren, 2000). In contrast to the findings reported here, recent studies with isolated islets showed that disruption of the M_3 receptor gene was

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sufficient to abolish muscarinic agonist-induced insulin (Duttaroy et al., 2004; Zawalich et al., 2004) and glucagon (Duttaroy et al., 2004) release. Analysis of pancreatic islets from M_1 receptor KO mice showed that muscarinic agonist-induced insulin release remained unaltered in the absence of M_1 receptors (D. Mears, C. Zimliki, and J. Wess, unpublished results). As already mentioned above, *in vivo* studies with mAChR mutant mice demonstrated that cholinergic stimulation of salivary flow is mediated by a mixture of M_1 and M_3 mAChRs (Gautam et al., 2004; Nakamura et al., 2004). Future studies will show whether M_1 receptors, together with M_3 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 M_1 and M_3 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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Figure legends

Fig. 1. Carbachol-induced increases in amylase secretion from dispersed pancreatic acini prepared from WT and M_1 and M_3 receptor single KO mice. Dispersed pancreatic acini were incubated with the indicated concentrations of carbachol for 30 min at 37 ^{o}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, as compared to the corresponding WT value.

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 M_1-M_4 mAChR transcripts in pancreatic acinar tissue from WT and M_1 and M_3 receptor single KO mice. No specific signal was obtained by the use of M_5 receptor-specific primers and probes, indicative of the absence of measurable levels of M_5 receptor mRNA. The sequences of the M_1-M_5 mAChR-selective TaqMan primers and probes have been reported previously (Gautam et al., 2004). Relative mAChR mRNA levels were expressed as ΔC_t values (C_t[mAChR]-C_t[internal control=18S rRNA]) (Livak and Schmittgen, 2001). Note that mAChR expression levels are similar in the presence or absence of functional M_1 or M_3 receptors. Data are presented as means ± S.D. from three different experiments (sets of mice), each carried out in duplicate.

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Fig. 3. Expression of M_1 and M_3 mAChR protein in mouse pancreatic acini studied by using an immunoprecipitation strategy. Membranes prepared from pancreatic acini from WT and M_1 and M_3 receptor KO mice were incubated with 2 nM of the non-selective muscarinic antagonist, [^{3}H]QNB. [^{3}H]QNB-labeled mAChRs were solubilized and immunoprecipitated with M_1 or M_3 receptor-selective antisera, as described under 'Materials and Methods'. The numbers of immunoprecipitated receptors are expressed as fmol [^{3}H]QNB binding sites per mg membrane protein. Data are given as means ± S.E.M. (five mice per group). **, $p < 0.05$.

Fig. 4. Effects of carbachol and bombesin on amylase secretion from dispersed pancreatic acini prepared from WT and M_1/M_3 receptor double KO mice. A, Carbachol-induced amylase secretion is abolished in acinar preparations from M_1/M_3 receptor double KO mice. B, Bombesin-induced amylase secretion remains unaffected by the lack of functional M_1 and M_3 mAChRs. Dispersed pancreatic acini were incubated with increasing concentrations of carbachol or bombesin for 30 min at 37 ^{o}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.

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Fig. 5. Histological profile of pancreatic exocrine tissue from WT and mAChR mutant mice. A, WT; B, M_1 receptor single KO; C, M_3 receptor single KO; D, M_1/M_3 receptor double KO mice. Sections were stained by hematoxylin/eosin and observed by light microscopy. Bar, 20 μm.

Fig. 6. Serum amylase levels of WT and mAChR mutant mice. 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, as compared to the corresponding WT value.

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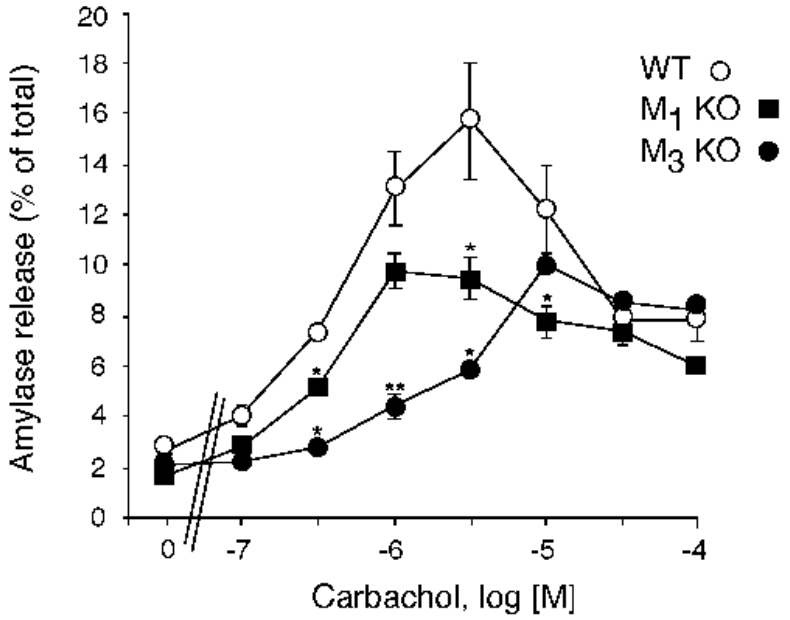

Fig. 1

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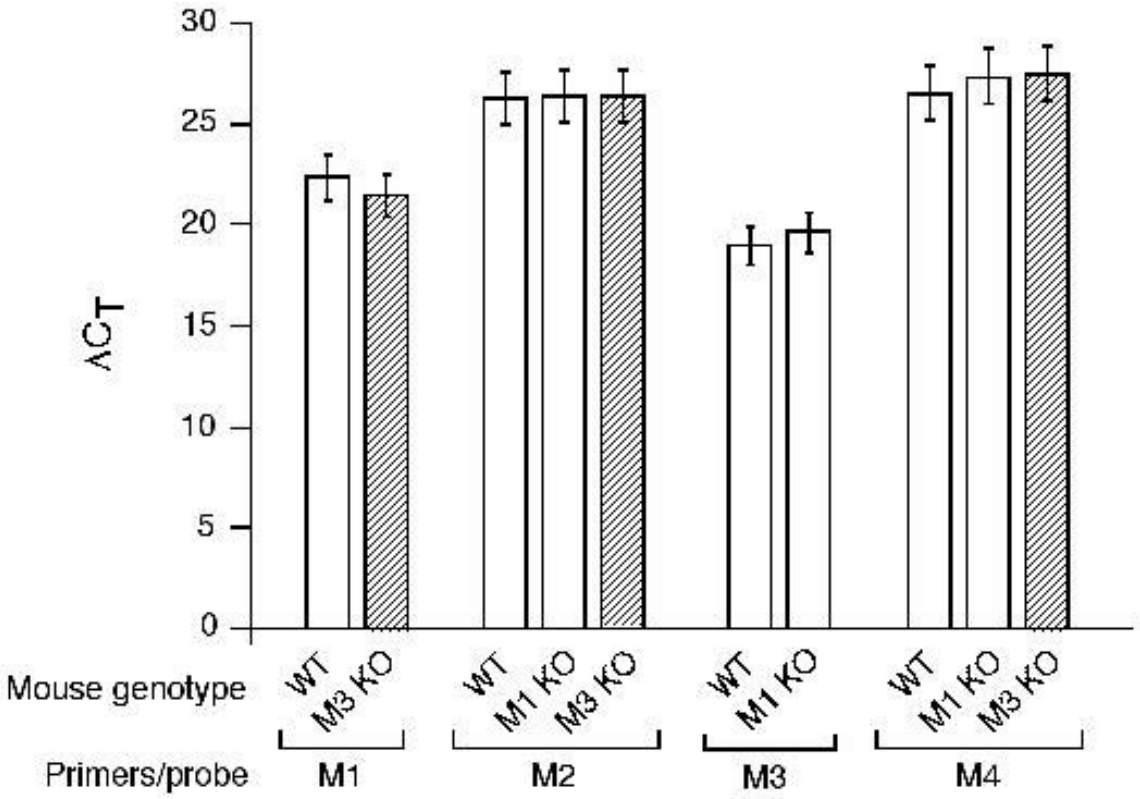

Fig. 2

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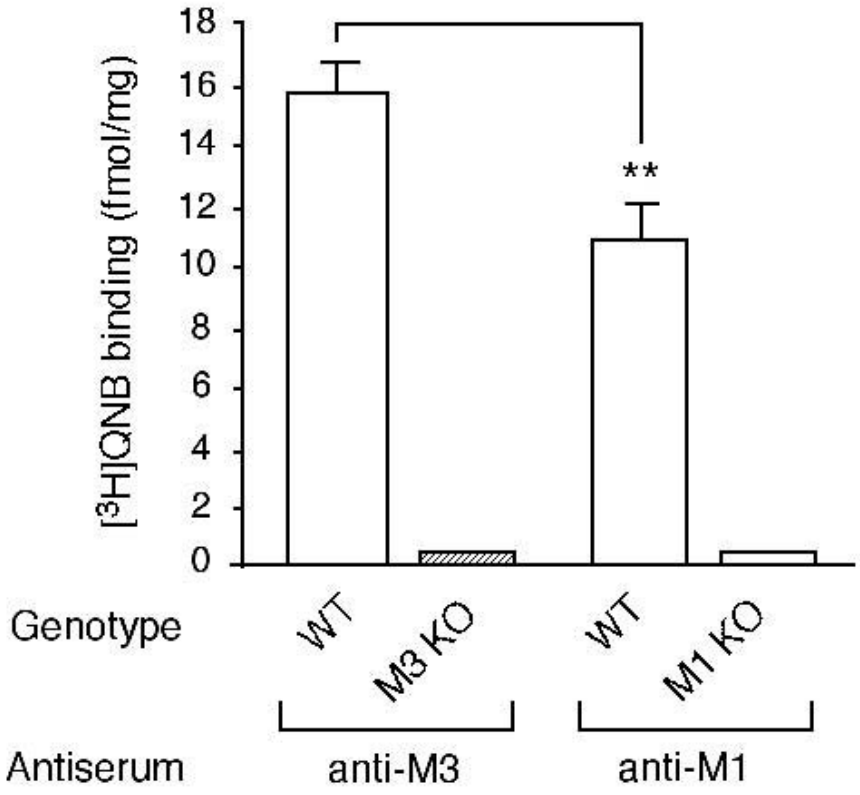

Fig. 3

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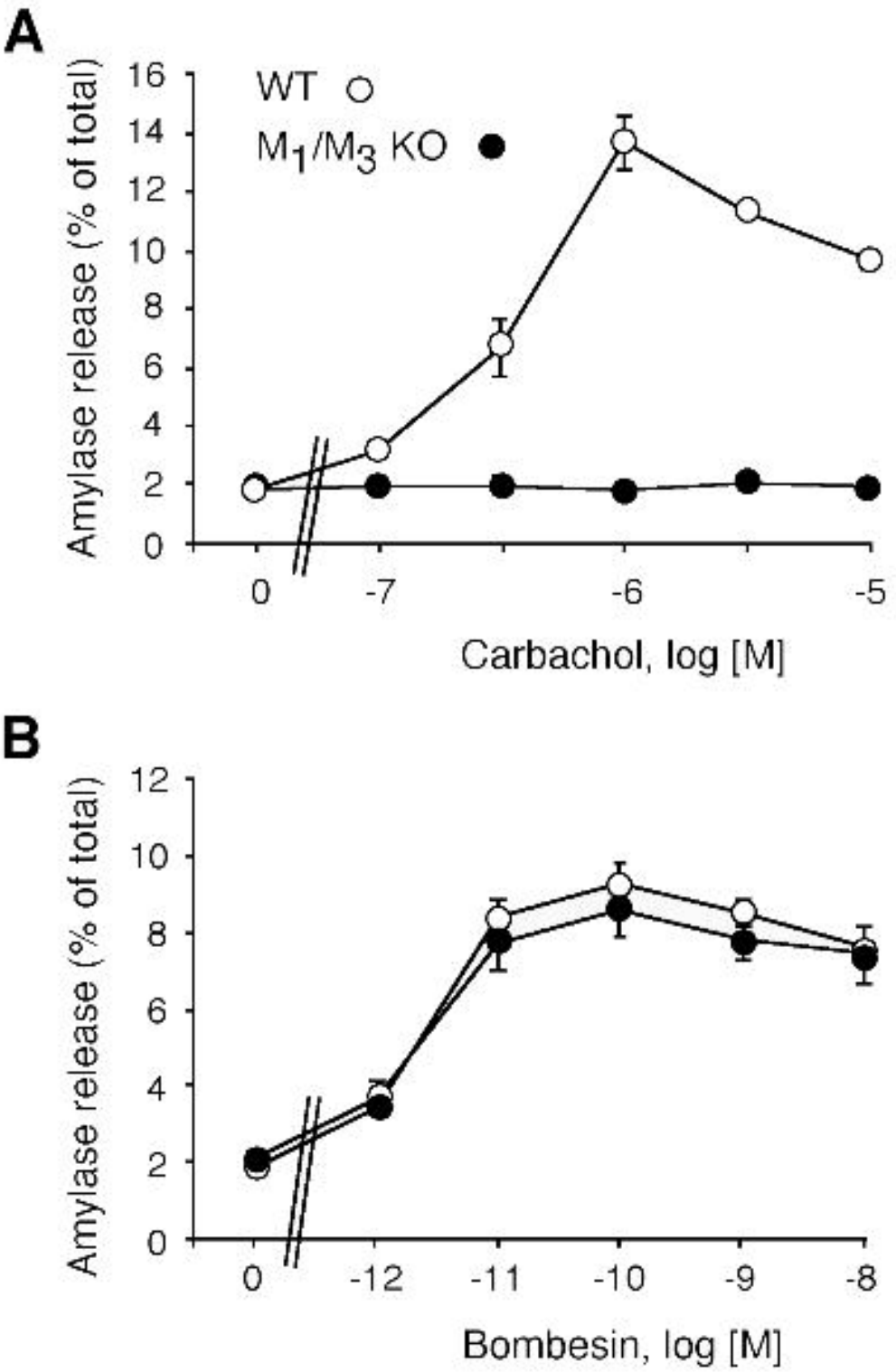

Fig. 4

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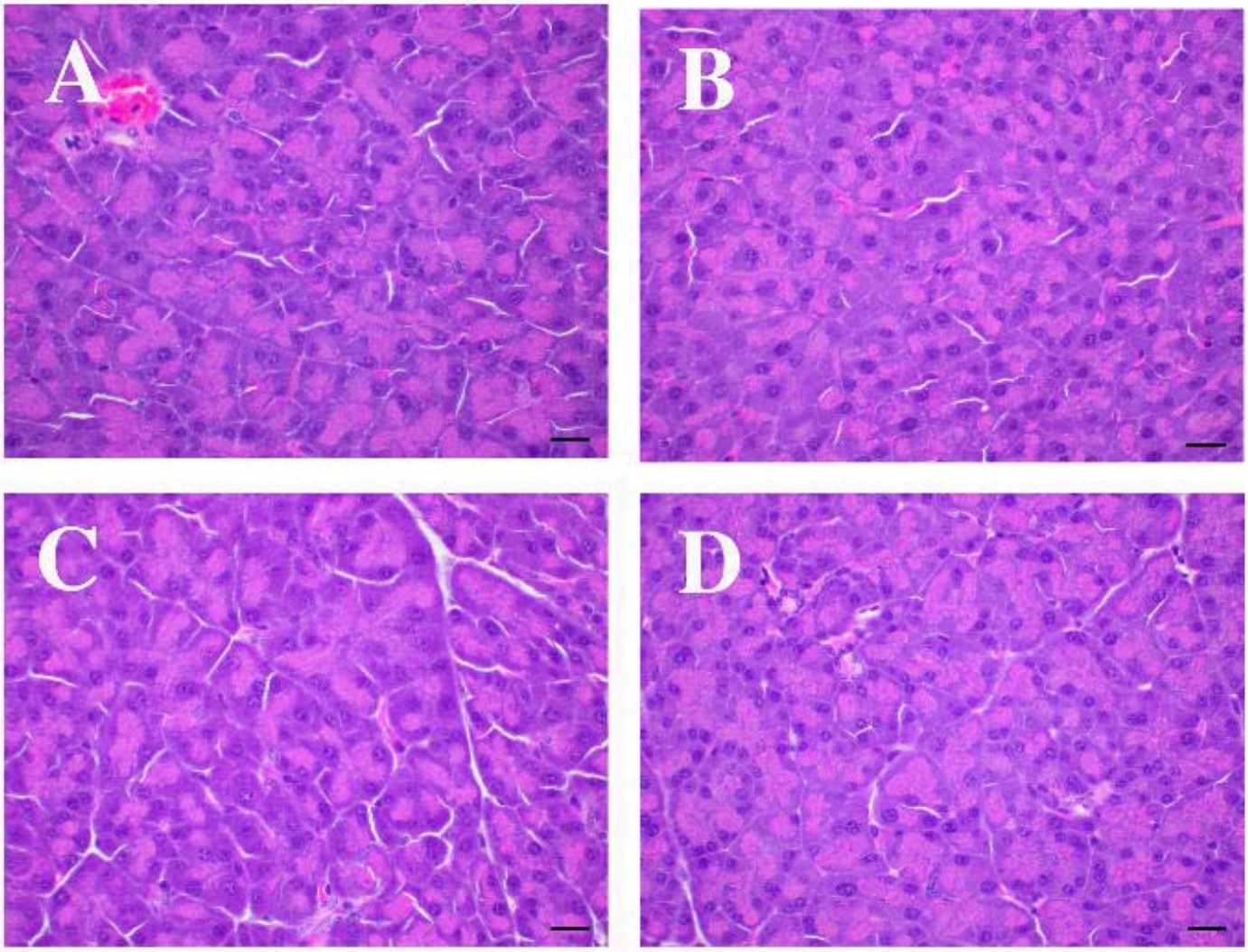

Fig. 5

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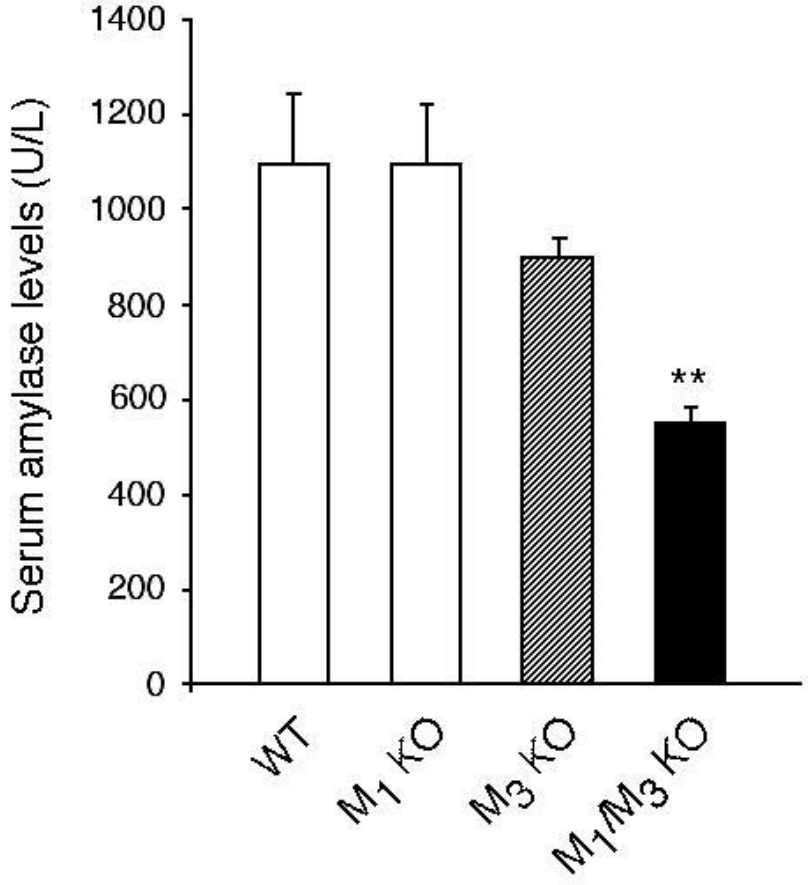

Fig. 6

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