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Cholinergic Stimulation of Salivary Secretion Studied with M_1 and M_3 Muscarinic Receptor Single- and Double-Knockout Mice

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ABSTRACT

Identification of the specific muscarinic acetylcholine receptor (mAChR) subtypes mediating stimulation of salivary secretion is of considerable clinical interest. Recent pharmacological and molecular genetic studies have yielded somewhat confusing and partially contradictory results regarding the involvement of individual mAChRs in this activity. In the present study, we re-examined the roles of M_1 and M_3 mAChRs in muscarinic agonist-mediated stimulation of salivary secretion by using M_1 and M_3 receptor single-knockout (KO) mice and newly generated M_1/M_3 receptor double-KO mice. When applied at a low dose (1 mg/kg, s.c.), the muscarinic agonist pilocarpine showed significantly reduced secretory activity in both M_1 and M_3 receptor single-KO mice. However, when applied at higher doses, pilocarpine induced only modestly reduced (5 mg/kg, s.c.) or unchanged (15 mg/kg, s.c.) salivation responses, respectively, in M_1 and M_3 receptor single-KO mice, indicating that the presence of either M_1 or M_3 receptors is sufficient to mediate robust salivary output. Quantitative reverse transcriptase-polymerase chain reaction studies with salivary gland tissue showed that the inactivation of the M_1 or M_3 mAChR genes did not lead to significantly altered mRNA levels of the remaining mAChR subtypes. Strikingly, the sialagogue activity of pilocarpine was abolished in M_1/M_3 receptor double-KO mice. However, salivary glands from M_1/M_3 receptor double-KO mice remained responsive to stimulation by the β-adrenergic receptor agonist, (*S*)-isoproterenol. Taken together these studies support the concept that a mixture of M_1 and M_3 receptors mediates cholinergic stimulation of salivary flow.

Saliva production and secretion are essential for maintaining the integrity of oral tissues, in speech, in protecting teeth from caries, and in the tasting and ingestion of food (Wiseman and Faulds, 1995). Xerostomia (oral dryness) is caused by reduced salivary flow associated with malfunction of salivary glands. Xerostomia can occur in response to radiation therapy to the head and neck region and frequently accompanies diseases such as diabetes insipidus and cardiac failure, emotional states such as fear and depression, and autoimmune diseases such as Sjögren's syndrome (Wiseman and Faulds, 1995). Moreover, a large number of drugs, including antidepressants, antihistamines, and antispasmodics, can cause xerostomia, primarily as a result of blockade of muscarinic receptors (Scully, 2003).

The major salivary glands, the submandibular, sublingual, and parotid glands, are innervated by both parasympathetic and sympathetic nerves (Baum, 1993). Activation of the parasympathetic fibers innervating the salivary glands leads to a copious flow of saliva, mediated by acetylcholine-induced activation of acinar cell muscarinic acetylcholine receptors (mAChRs) (Baum, 1993). In fact, mAChR-mediated stimulation of saliva output is considered the major mechanism by which salivary fluid secretion is regulated physiologically (Baum, 1993; Turner and Sugiya, 2002; Scully, 2003).

Stimulation of mAChRs located on salivary acinar cells is known to lead to the activation of G proteins of the G_q family (Baum, 1993; Luo et al., 2001) which mediate an increase in intracellular calcium levels and the opening of calcium-activated Cl^- and K^+ channels (Baum, 1993; Turner and Sugiya, 2002). Recent studies with gene knockout (KO) mice demonstrated that muscarinic agonist-induced salivary secretion is strongly reduced in mice lacking the aquaporin-5 water channel (Ma et al., 1999) or the $Na^+/K^+/2Cl^-$ cotransporter (Evans et al., 2000), indicating that these two proteins represent key components of the signaling pathway stimulating salivary flow.

ABBREVIATIONS: mAChR, muscarinic acetylcholine receptor; KO, knockout; WT, wild type; RT, reverse transcriptase; PCR, polymerase chain reaction; C_t, threshold cycle; NMS, *N*-methyl scopolamine.

Identification of the specific mAChR subtype(s) mediating stimulation of salivary secretion is of considerable interest for the development of more selective muscarinic sialagogues or of drugs with reduced side effects. The mAChR family consists of five molecularly distinct members (M_1-M_5) that can be subdivided, based on their G protein-coupling profiles, into two major functional subclasses (Caulfield, 1993; Wess, 1996; Caulfield and Birdsall, 1998). Whereas the odd-numbered receptors (M_1, M_3, and M_5) are selectively coupled to G proteins of the G_q family, the even-numbered mAChR subtypes (M_2 and M_4) are preferentially linked to G proteins of the G_i class (Caulfield, 1993; Wess, 1996; Caulfield and Birdsall, 1998).

Pharmacological, biochemical, and molecular genetic studies indicate that the M_3 receptor subtype plays a key role in mediating increased salivary flow (Laniyonu et al., 1990; Dai et al., 1991; Caulfield, 1993; Watson et al., 1996; Moriya et al., 1999; Matsui et al., 2000; Bockman et al., 2001; Bymaster et al., 2003). However, pharmacological and biochemical studies suggest that M_1 (Watson and Culp, 1994; Culp et al., 1996; Luo et al., 2001; Tobin et al., 2002) and M_5 mAChRs (Flynn et al., 1997; Meloy et al., 2001; Tobin et al., 2002) may also play a role in muscarinic agonist-mediated salivary secretion. Moreover, two recent studies reported that muscarinic agonist-induced stimulation of salivary output was impaired not only in M_3 receptor KO mice but also in M_1 and M_4 receptor KO mice (Bymaster et al., 2003) and M_5 receptor KO mice (Takeuchi et al., 2002). Taken together, these studies suggest that multiple mAChRs (M_1, M_3, M_4, and M_5) may be involved in cholinergic stimulation of salivary flow.

To learn more about the functional roles of the M_1 and M_3 receptor subtypes as well as of non-M_1/M_3 receptors in cholinergic stimulation of salivary secretion in vivo, we used M_1 and M_3 receptor single-KO mice and newly generated M_1/M_3 receptor double-KO mice as novel experimental tools. All mutant mice and the corresponding WT controls received three different doses (1, 5, and 15 mg/kg, s.c.) of the nonselective muscarinic agonist pilocarpine, and the magnitudes of the resulting salivation responses were recorded. These studies were complemented by radioligand binding and quantitative reverse transcriptase (RT)-PCR studies (TaqMan), primarily to exclude the possibility that inactivation of one specific mAChR gene causes altered expression levels of the remaining mAChR subtypes.

Whereas high doses of pilocarpine led to robust salivary flow in M_1 and M_3 receptor single-KO mice, pilocarpine-induced salivary secretion was abolished in M_1/M_3 receptor double-KO mice. This finding is consistent with the concept that mAChR-mediated stimulation of salivary secretion is mediated by a mixture of M_1 and M_3 receptors and that other glandular mAChRs are unlikely to contribute to this activity to a significant extent. These observations should be of considerable relevance for the design of more selective sialagogues or of muscarinic and nonmuscarinic drugs with reduced side effects on salivary flow.

Materials and Methods

Animals. The generation of homozygous M_1 and M_3 receptor KO mice (129SvEv [50%] × CF1 [50%]) has been described previously (Yamada et al., 2001; Miyakawa et al., 2001; Fisahn et al., 2002). To generate mice deficient in both M_1 and M_3 mAChRs, homozygous M_1 receptor KO mice were mated with homozygous M_3 receptor KO mice. The resulting F1 compound heterozygotes were then intercrossed to generate F2 offspring. According to Mendelian inheritance, 1/16 of the F2 pups would be expected to be homozygous for both the M_1 and the M_3 receptor gene disruptions (M_1/M_3 receptor double-KO mice). M_1/M_3 receptor double-KO mice were obtained at a frequency of about 4.7%, which is slightly below the predicted Mendelian frequency of 6.25%. The M_1/M_3 receptor double-KO mice were interbred to generate mice used for the experiments described in this study. In parallel, the wild-type (WT) F2 mice were interbred to obtain WT control mice. Thus, both M_1/M_3 receptor double-KO mice and the corresponding WT control mice had a genetic background that was identical to that of the M_1 and M_3 single-KO mice (129SvEv [50%] × CF1 [50%]). The identity of the various mutant mouse strains was verified by Southern blotting and PCR analysis of mouse tail DNA as described previously (Yamada et al., 2001; Fisahn et al., 2002).

The generation of M_2/M_3 (Struckmann et al., 2003) and M_2/M_4 (Duttaroy et al., 2002) receptor double-KO mice has been described previously. These mutant mice had the following mixed genetic background: 129J1 [50%] × CF1 [50%]. M_1/M_4 receptor double-KO mice were generated in a fashion identical to that described above for the M_1/M_3 receptor double-KO mice (J. Wess, unpublished results). The M_1/M_4 receptor double-KO mice had the same mixed genetic background as the M_1/M_3 receptor double-KO mice (129SvEv [50%] × CF1 [50%]).

All experiments were performed during the light cycle using adult male mice that were 10 to 18 weeks old. In all experiments, KO mice and age-matched WT mice of the same genetic background were run in parallel. All animal studies were conducted according to the National Institute of Diabetes and Digestive and Kidney Diseases guidelines for standard animal care and usage.

Salivation Studies. For salivation studies, WT and mAChR mutant mice were anesthetized with pentobarbital (50 mg/kg, i.p.). Salivation responses to pilocarpine hydrochloride (1, 5, and 15 mg/kg, s.c.) or (*S*)-isoproterenol bitartrate (1 mg/kg, s.c.) (both compounds were purchased from Sigma) were quantitated in 5-min intervals over a 35-min observation period by collecting saliva on filter paper using a method similar to that described by Parkes and Parks (1972). In brief, mice were placed face down on a slightly inclined surface (about 5° to the horizontal), with the heads facing downwards to allow free flow of saliva. After the injection of secretagogues, a preweighed piece of circular Whatman filter paper (diameter, 125 mm) was placed immediately below the mouth area of each mouse. Fresh pieces of preweighed filter paper were added in 5-min intervals (last change of filter paper was 30 min after injection of the secretagogue). Filter papers were weighed for the quantitation of saliva production.

Radioligand Binding Studies. Submandibular salivary glands were removed from individual mice and frozen immediately in liquid nitrogen. Tissue samples were homogenized for 30 s in 50 volumes of binding buffer (50 mM sodium phosphate buffer, pH 7.4, containing 2 mM $MgCl_2$, and 100 mM NaCl) by using a Brinkmann homogenizer. The homogenates were then filtered through four layers of cheesecloth and centrifuged once at 50,000 × *g*. The membrane pellets were resuspended in binding buffer at 60 mg/ml (tissue wet weight) and binding assays were carried out in triplicate according to a published method (Bymaster et al., 2003). In brief, membrane homogenates (~0.4 mg of protein/tube) were incubated in a 0.2-ml total volume of binding buffer for 2 h at room temperature (22°C). The non–subtype-selective muscarinic antagonist [^{3}H]*N*-methylscopolamine ([^{3}H]NMS; specific activity, 83.5 Ci/mmol; PerkinElmer Life and Analytical Sciences, Boston, MA), served as a radioligand (final concentration in most assays was 3 nM). In saturation binding assays using submandibular gland membranes prepared from WT mice, six different concentrations of [^{3}H]NMS (0.02 to 3 nM) were tested. Binding data from saturation bindings studies were analyzed

using the nonlinear curve-fitting program Prism 3.0 (GraphPad Software, San Diego, CA).

Nonspecific binding was determined in the presence of 10 μM atropine. Bound and free ligand were separated by vacuum filtration over GF/B filters (Whatman, Clifton, NJ), pretreated with 0.3% polyethylenimine for 1 h. The filters were washed three times with 5 ml of ice-cold phosphate buffer, dried, and placed in vials with 7 ml of Bio-Safe II scintillation cocktail (Research Products International, Mt. Prospect, IL). Radioactivity bound to the filters was determined after 18 h of extraction.

Quantitative Real-Time PCR (TaqMan) Analysis of Salivary Gland cDNA from WT and mAChR Mutant Mice. Three age-matched WT, M_1 receptor KO, and M_3 receptor KO mice were sacrificed, and the sublingual and submandibular glands were excised and immediately snap frozen in liquid nitrogen. Total RNA was obtained from the individual gland tissues by homogenization in TRIzol (Invitrogen) according the manufacturer's instructions. The RNA preparations were further processed using the RNA cleanup protocol of the RNeasy Mini Kit (QIAGEN, Valencia, CA), including on-column DNase treatment using the RNase-free DNase set (QIAGEN), to obtain total RNA free of contaminating genomic DNA. RNA quantity and quality were assessed by measuring the absorbance at 260 and 280 nm using an Eppendorf Biophotometer (Eppendorf–5 Prime, Inc., Boulder, CO). Salivary gland 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) according to 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 mRNAs in each reaction. Specific mouse M_1–M_5 receptor primers and TaqMan probes (Table 1) were designed from GenBank sequences using Primer Express 1.0 software (Applied Biosystems) and were synthesized by Applied Biosystems. The subtype specificity of the primers/probes used was verified in control reactions using plasmid DNA containing the coding sequences of the mouse M_1–M_5 mAChRs (data not shown). The amplification efficiencies of the individual primer-probe pairs were determined by serial dilution of cDNA and plotting of threshold cycle (C_t) values versus log input amount of cDNA. The amplification efficiencies of all primer-probe pairs were found to be between 1.9 and 2.0 (data not shown).

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 following cycling conditions were used: 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. Control reactions using RNA samples that had not been treated with RT were included as negative controls, confirming that the RNA samples were free of contaminating genomic DNA (data not shown). All RT reactions and TaqMan assays were carried out in duplicate. Relative mAChR expression levels were compared between samples from WT versus M_1 or M_3 receptor KO mice by comparing ΔC_t values (C_t[mAChR] − C_t[control=18S rRNA]) (Livak and Schmittgen, 2001).

Morphological Studies. Salivary glands were harvested from WT and M_1/M_3 receptor double-KO 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 (BX41; Olympus, Tokyo, Japan). Photographs were taken with an Olympus DP12 digital camera, using the 20× oil immersion lens.

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

Results

Dose-Dependence of the Sialagogue Activity of Pilocarpine in M_1 and M_3 Receptor Single-KO Mice. At first, we examined the ability of three different doses of pilocarpine (1, 5, and 15 mg/kg, s.c.) to stimulate salivary flow in M_1 and M_3 receptor single-KO mice (Fig. 1). Administration of the lowest dose of pilocarpine (1 mg/kg) resulted in a significant reduction in saliva output in both M_1 and M_3 receptor single-KO mice, compared with the corresponding WT control mice. The reduction in the magnitude of salivary secretion was clearly more pronounced in the M_3 receptor KO than in the M_1 receptor KO mice (reduction in saliva output by ~90 and ~50%, respectively, measured over the entire 35 min observation period; Fig. 1A).

In contrast, administration of the intermediate pilocarpine

TABLE 1

Nucleotide sequences of TaqMan PCR primers and probes used in real-time RT-PCR experiments to quantify relative M_1-M_5 mAChR (mouse) expression levels

All probes were labeled at the 5′ end with 6-carboxyfluorescein and at the 3′ end with 6-carboxytetramethylrhodamine. Nucleotide positions refer to the receptor coding sequences

Mouse mAChR gene	TaqMan PCR Primer and Probe Sequences	Genbank Accession No.	Nucleotide Positions
M_1			
Sense	5′-CAGAAGTGGTGATCAAGATGCCTAT-3′	J04192	893–917
Probe	5′-TAGATCCTGAGGCACAGGCACCCAC-3′		920–944
Antisense	5′-GAGCTTTTGGGAGGCTGCTT-3′		946–965
M_2			
Sense	5′-TGGAGCACAACAAGATCCAGAAT-3′	AF264049	764–786
Probe	5′-CAAGGCTCTGCGGGACGGTGG-3′		789–809
Antisense	5′-CCCCTGAACGCAGTTTTCA-3′		813–831
M_3			
Sense	5′-CCGCTCTACCTCTGTCCTTCA-3′	AF264050	1352–1372
Probe	5′-AAGCCACGCTGGCTAAGAGGTTTGC-3′		1376–1400
Antisense	5′-GGTGATCTGACTTCTGGTCTTGAG-3′		1402–1425
M_4			
Sense	5′-GTGACTGCCATCGAGATCGTAC-3′	X63473	1225–1246
Probe	5′-CCAGCTGGTATGCGCCCAGCAG-3′		1255–1276
Antisense	5′-CAAACTTTCGGGCCACATTG-3′		1278–1297
M_5			
Sense	5′-GGCCCAGAGAGAACGGAAC-3′	AF264051	783–801
Probe	5′-AGGCCTCCTGGTCATCCTCCCGTA-3′		803–826
Antisense	5′-TTCCCGTTGTTGAGGTGCTT-3′		828–847

dose (5 mg/kg) produced only a rather modest reduction in salivary flow in the M_1 and M_3 receptor single-KO mice (reduction in saliva output by ~20% measured over the entire 35-min observation period; Fig. 1B). This decrease proved to be statistically significant only in the case of the M_3 receptor KO mice (Fig. 1B). Finally, the highest pilocarpine dose used (15 mg/kg) induced robust salivation responses in both M_1 and M_3 receptor single-KO mice that did not differ significantly from the corresponding WT responses (Fig. 1C).

Analysis of mAChR Gene Expression in Mouse Salivary Glands by Quantitative Real-Time PCR (TaqMan). As described in the previous paragraph, pilocarpine (5 and 15 mg/kg) retained the ability to mediate robust salivation responses in M_1 and M_3 receptor single-KO mice (Fig. 1). To exclude the possibility that the robustness of these responses was caused by altered mAChR gene expression patterns (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), we used quantitative real-time PCR (TaqMan) to determine the relative expression levels of the M_1-M_5 receptor transcripts in salivary glands from WT and M_1 and M_3 receptor single-KO mice (for experimental details, see *Materials and Methods*). 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).

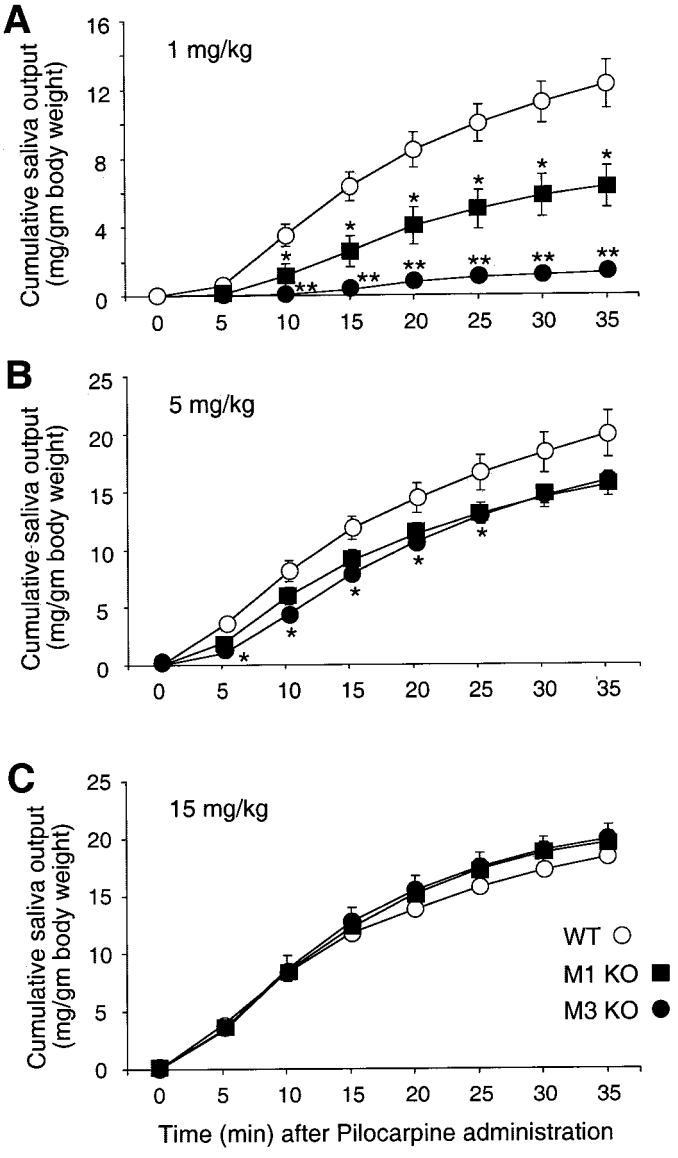

Fig. 1. Pilocarpine-induced salivation in WT and M_1 and M_3 receptor single-KO mice. Pilocarpine (A, 1 mg/kg; B, 5 mg/kg; C, 15 mg/kg) was administered s.c. to WT and M_1 and M_3 receptor single-KO mice. Cumulative salivary secretion for the entire observation period was measured for up to 35 min after pilocarpine administration, as described under *Materials and Methods*. Data are given as means ± S.E.M. (five or six mice per group). *, $P < 0.05$; **, $P < 0.001$, compared with the corresponding WT value.

Quantitative real-time PCR analysis of cDNA prepared from mouse submandibular glands indicated that M_1 receptor transcripts were present at similar levels in WT and M_3 receptor KO mice (Fig. 2). Likewise, M_3 receptor transcripts were expressed at similar levels in WT and M_1 receptor KO mice (Fig. 2). In addition, TaqMan analysis showed that submandibular gland tissue from WT mice also contained low levels of M_2, M_4, and M_5 receptor mRNA (note the significant increase in ΔC_t values compared with the corresponding M_1 and M_3 receptor values). The relative mRNA expression levels of the M_2, M_4, and M_5 receptors remained unaffected by the lack of M_1 or M_3 receptors (Fig. 2). Very similar results were obtained using cDNA prepared from sublingual gland tissue from WT and M_1 and M_3 receptor KO mice (data not shown). Taken together, these data indicate that inactivation of the M_1 or M_3 mAChR genes does not result in significantly altered mRNA levels of the remaining mAChRs expressed in salivary gland tissue.

Generation of M_1/M_3 mAChR Double-KO Mice. To test the hypothesis that pilocarpine-induced salivary secretion is mediated by a mixture of M_1 and M_3 mAChRs, we generated M_1/M_3 receptor double-KO mice as novel experimental tools (for experimental details, see *Materials and Methods*). The M_1/M_3 receptor double-KO mice were fertile, seemed healthy, and showed no obvious behavioral abnormalities. However, like the M_3 receptor single-KO mice (Yamada et al., 2001),

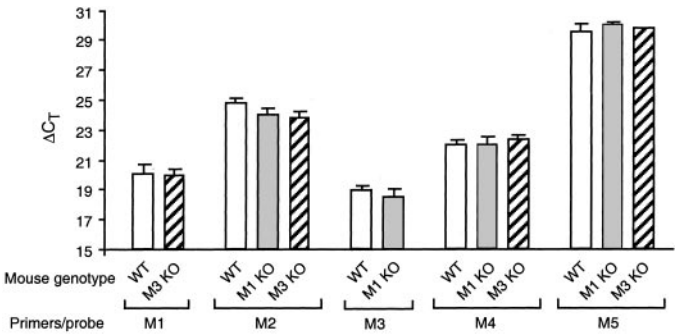

Fig. 2. Quantitative RT-PCR analysis (TaqMan) of M_1-M_5 mAChR expression in submandibular gland tissue from WT and mAChR mutant mice. Quantitative RT-PCR analysis (TaqMan) was used to determine the relative levels of M_1-M_5 mAChR transcripts in submandibular gland tissue from WT and M_1 and M_3 receptor single-KO mice. The sequences of the receptor subtype-selective TaqMan primers and probes used are given in Table 1. Relative mAChR expression levels were expressed as ΔC_t values (C_t[mAChR]-C_t[internal control=18S rRNA]) (Livak and Schmittgen, 2001). Comparison of ΔC_t values indicates that mAChR expression levels were not significantly affected by the lack of M_1 or M_3 receptors. Very similar results were obtained with cDNA prepared from sublingual gland tissue from WT and M_1 and M_3 receptor KO mice (data not shown). Data are presented as means ± S.D. from three different experiments (sets of mice), each carried out in duplicate.

adult M_1/M_3 receptor double-KO mice weighed significantly less (~30–40%) than the corresponding WT control mice. We also noted that intermating of male and female M_1/M_3 receptor double-KO mice resulted in somewhat reduced litter sizes (average number of pups per litter: WT, 8.4 ± 0.9; M_1/M_3 double KO, 5.4 ± 0.7; $n = 14$). The reason underlying this phenomenon remains unclear at present.

Pilocarpine-Induced Salivary Secretion Is Abolished in M_1/M_3 Receptor Double-KO Mice. We next tested the ability of pilocarpine (1, 5, and 15 mg/kg, s.c.) to stimulate salivary outflow in M_1/M_3 receptor double KO and their corresponding WT control mice. These studies showed that pilocarpine-mediated salivary secretion was essentially abolished in M_1/M_3 receptor double-KO mice (Fig. 3), independent of the pilocarpine dose used. However, (*S*)-isoproterenol (1 mg/kg, s.c.), an agent that stimulates salivary output via activation of β-adrenergic receptors, retained its stimulatory effect on saliva production in M_1/M_3 receptor double-KO mice (Fig. 4), indicating that the absence of both M_1 and M_3 mAChRs did not interfere with the integrity of downstream signal transduction cascades triggering salivary secretion. In fact, (*S*)-isoproterenol-mediated saliva outflow was even slighter higher in M_1/M_3 receptor double-KO mice than in WT mice at several time points ($p < 0.05$; Fig. 4).

In addition, light microscopic studies showed that the morphology of submandibular, sublingual, and parotid salivary glands from M_1/M_3 receptor double-KO mice did not differ from that of the corresponding WT control mice (Fig. 5).

[^{3}H]NMS Binding Studies with Salivary Gland Tissue. We next performed [^{3}H]NMS binding studies to estimate the densities of M_1 and M_3 receptors expressed by mouse salivary gland tissue. In particular, we labeled membranes prepared from submandibular glands from M_1 and M_3 receptor single KO and M_1/M_3 receptor double-KO mice and their corresponding WT strains with a high concentration (3 nM) of the non–subtype-selective muscarinic antagonist [^{3}H]NMS. In gland tissue from WT mice, [^{3}H]NMS labeled 351 ± 51 fmol of receptors/mg of membrane protein (the WT receptor density was set equal to 100% in each individual experiment; Fig. 6). The number of detectable [^{3}H]NMS binding sites was reduced by ~25% and ~65% in M_1 and M_3 receptor single-KO mice, respectively (Fig. 6), suggesting that ~25% of all mAChRs present in the mouse submandibular gland represent M_1 and ~65% represent M_3 receptors. Only residual [^{3}H]NMS binding activity (~8% of WT binding) remained in gland tissue from M_1/M_3 receptor double-KO mice (Fig. 6).

Saturation binding studies with membranes prepared from submandibular glands from WT mice showed that [^{3}H]NMS bound to submandibular gland mAChRs with a K_D of 0.357 ± 0.007 nM ($n = 3$). The [^{3}H]NMS concentration of 3 nM used for the studies described above therefore labels about 90% of the total mAChR population.

Pilocarpine-Induced Salivation in M_1/M_4, M_2/M_3, and M_2/M_4 Receptor Double-KO Mice. Besides the M_1/M_3 receptor double-KO mice described in detail above, we recently also generated M_2/M_3 (Struckmann et al., 2003), M_2/M_4 (Dut-

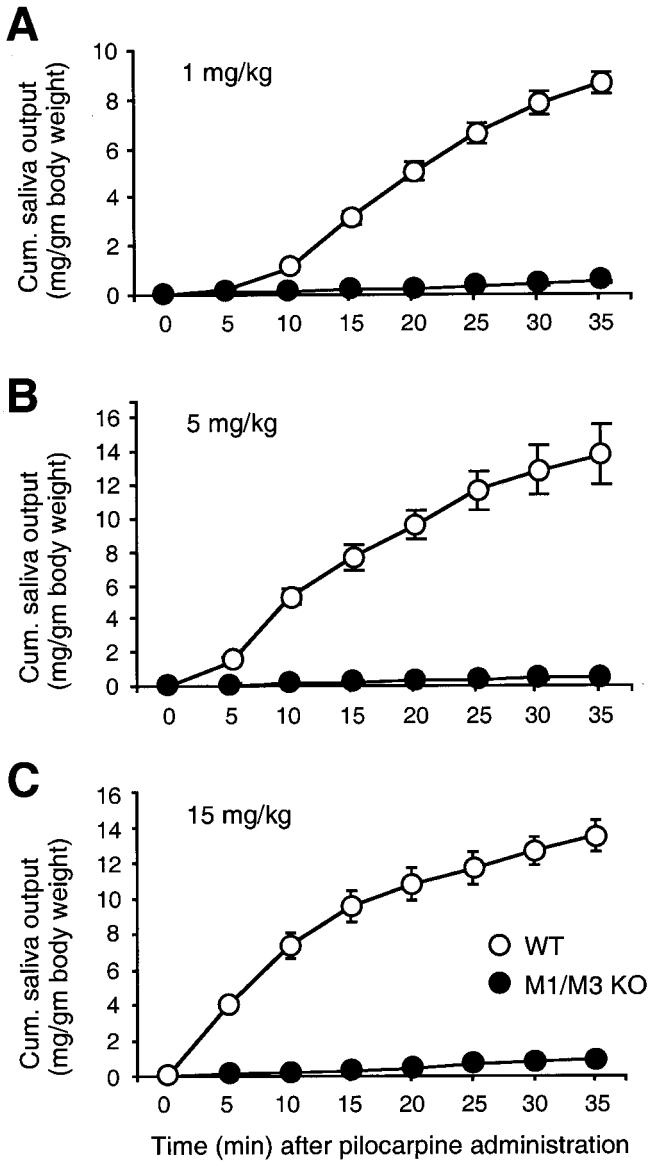

Fig. 3. Pilocarpine-induced salivation in WT and M_1/M_3 receptor double-KO mice. Pilocarpine (A, 1 mg/kg; B, 5 mg/kg; C, 15 mg/kg) was administered s.c. to WT and M_1/M_3 receptor double-KO mice. Cumulative salivary secretion for the entire observation period was measured for up to 35 min after pilocarpine administration, as described under *Materials and Methods*. Note that pilocarpine failed to stimulate salivary secretion in M_1/M_3 receptor double-KO mice. Data are given as means ± S.E.M. (five to eight mice per group).

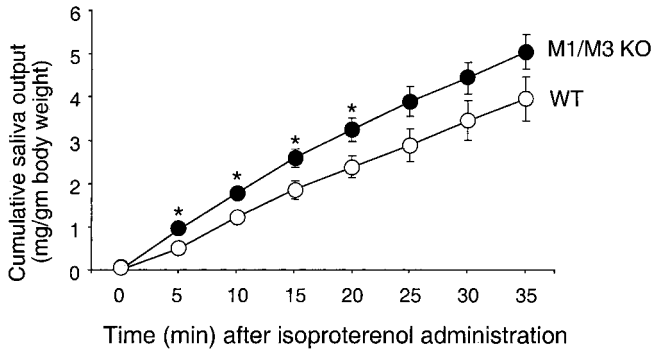

Fig. 4. (*S*)-Isoproterenol-induced salivation in WT and M_1/M_3 receptor double-KO mice. (*S*)-Isoproterenol (1 mg/kg, s.c.) was administered to WT and M_1/M_3 receptor double-KO mice. Cumulative salivary secretion for the entire observation period was measured for up to 35 min after drug administration, as described under *Materials and Methods*. Data are given as means ± S.E.M. (five or six mice per group; *, $P < 0.05$, compared with the corresponding WT value).

taroy et al., 2002), and M_1/M_4 receptor double-KO mice (J. Wess, unpublished results). As shown in Fig. 7, pilocarpine (15 mg/kg, s.c.) induced the flow of similar amounts of saliva in M_1/M_4, M_2/M_3, and M_2/M_4 receptor double-KO mice, compared with their corresponding WT control mice (saliva output was measured over the entire 35-min observation period).

Discussion

Muscarinic antagonists are used clinically in many pathophysiological conditions including, for example, the treatment of Parkinson's disease and the reduction of increased bronchial, gastrointestinal, and bladder smooth muscle tone (Eglen et al., 1999; Wess, 2001). A major side effect of these drugs is impaired salivary flow (dry mouth), predicted to be caused by blockade of glandular mAChRs. Impaired salivary flow is also a frequent side effect of many other classes of drugs including the tricyclic antidepressants, antipsychotics, β-adrenergic receptor blockers, and antihistaminergic drugs (Scully, 2003). Muscarinic agonists such as pilocarpine are successfully used in the treatment of xerostomia (Wiseman and Faulds, 1995; Fox et al., 2001). Thus, determination of the specific mAChR subtypes involved in mediating enhanced salivary flow is of considerable therapeutic interest.

To learn more about the functional roles of the M_1 and M_3 mAChR subtypes in muscarinic agonist-induced salivary secretion in vivo, we used M_1 and M_3 receptor single-KO mice and newly generated M_1/M_3 receptor double-KO mice as novel experimental tools. All mutant mice and the corresponding WT controls received three different doses (1, 5, and 15 mg/kg, s.c.) of the nonselective muscarinic agonist pilocarpine.

Studies with M_1 and M_3 receptor single-KO mice showed that the magnitudes of the observed salivation responses were critically dependent on the administered pilocarpine dose. The salivation response to the lowest dose of pilocarpine (1 mg/kg) was drastically reduced (by ~90%), but not abolished, in M_3 receptor single-KO mice (Fig. 1A). A similar effect was observed when M_3 receptor single-KO mice were treated with another muscarinic agonist, oxotremorine (0.3 mg/kg, s.c.) (Bymaster et al., 2003). In contrast, Matsui et al. (2000) reported that pilocarpine (1 mg/kg, s.c.)-mediated salivary secretion was completely abolished in M_3 receptor single-KO mice. A possible reason for the discrepancy between this and our study may be that Matsui et al. (2000) used a different method to measure salivary flow and employed mice of a somewhat different genetic background. It should also be noted that we found previously that 1 mg/kg (s.c.) of pilocarpine had little sialagogue activity (<2 mg saliva/g measured over a 30-min observation period) in either WT or M_3 receptor KO mice (Yamada et al., 2001). Possible explanations for the relatively low pilocarpine sensitivity of the mice used in this previous study may include differences in mouse age (present study, 10 weeks; previous study, 24 weeks) or

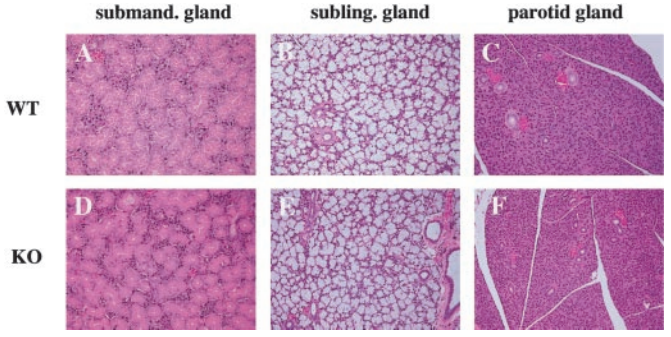

Fig. 5. Histological profile of salivary gland tissues from WT and M_1/M_3 receptor double-KO mice. Sections of submandibular, sublingual, and parotid glands from WT (A–C) and M_1/M_3 double-KO mice (D–F) were stained by hematoxylin/eosin and observed by light microscopy (20×).

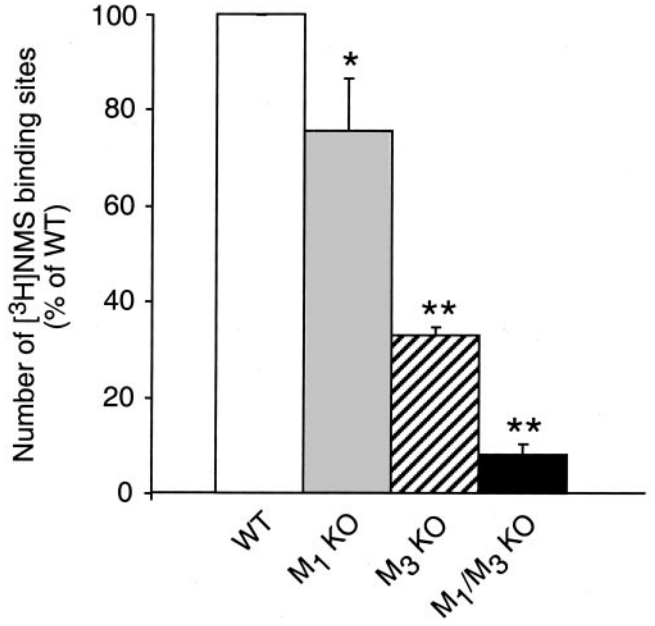

Fig. 6. Number of [^{3}H]NMS binding sites in submandibular gland tissue from WT and mAChR KO mice. Membranes were prepared from submandibular glands of M_1 receptor KO, M_3 receptor KO, and M_1/M_3 receptor double-KO mice and the corresponding WT control mice. Membranes were then incubated with 3 nM of the non–subtype-selective muscarinic antagonist [^{3}H]NMS, as described under *Materials and Methods*. In each individual experiment, the number of binding sites observed with the WT mice was set equal to 100% (absolute number of binding sites, 351 ± 51 fmol/mg; $n = 6$). Data are presented as means ± S.E.M. (three to five mice per KO group). *, $P < 0.05$; **, $P < 0.01$ compared with WT.

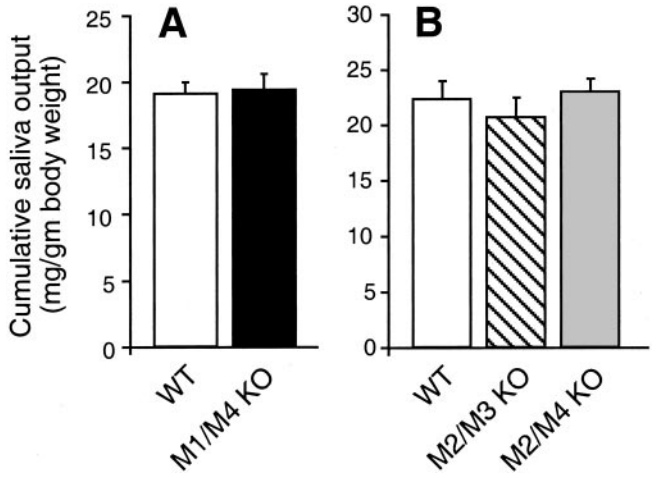

Fig. 7. Pilocarpine-induced salivation in WT and M_1/M_4, M_2/M_3, and M_2/M_4 receptor double-KO mice. Pilocarpine (15 mg/kg) was administered s.c. to M_1/M_4 receptor double-KO mice and the corresponding WT control mice (A) and to M_2/M_3 and M_2/M_4 receptor double-KO mice and the corresponding WT control mice (B). The amount of saliva secreted over the entire 35-min observation period was measured as described under *Materials and Methods*. Data are given as means ± S.E.M. (four mice per group).

mouse genetic background (present study, 129SvEv × CF1; previous study, 129SvEV × C57BL/6J). In any case, our present results are consistent with the concept that the M_3 receptor plays a key role in mediating cholinergic stimulation of salivary secretion (Laniyonu et al., 1990; Dai et al., 1991; Caulfield, 1993; Watson et al., 1996; Moriya et al., 1999; Matsui et al., 2000; Bockman et al., 2001; Bymaster et al., 2003).

It is interesting that administration of the lowest dose of pilocarpine (1 mg/kg) also resulted in a significant reduction (by ~50%) in salivary output in M_1 receptor single-KO mice (Fig. 1A), indicating that M_1 receptor activation also contributes to cholinergic stimulation of salivary flow. Likewise, Bymaster et al. (2003) recently reported that oxotremorine (0.3 mg/kg, s.c.) also showed significantly reduced sialagogue activity in M_1 receptor KO mice (reduction in saliva output by ~35% measured over a 15-min observation period). Pharmacological and biochemical techniques have also shown that M_1 receptors (as well as M_3 receptors) mediate stimulation of mucous acinar exocrine secretion in rat sublingual glands (Culp et al., 1996; Luo et al., 2001).

In contrast with the lowest pilocarpine dose used (1 mg/kg, s.c.), the intermediate pilocarpine dose employed (5 mg/kg, s.c.) produced only a modest reduction (by ~20%) in salivary flow in the M_1 and M_3 receptor KO mice, which proved to be statistically significant only in the case of the M_3 receptor KO mice (Fig. 1B). Finally, the highest pilocarpine dose used (15 mg/kg, s.c.) induced WT-like salivation responses in both M_1 and M_3 receptor single-KO mice (Fig. 1C), indicating that the presence of either M_1 or M_3 receptors is sufficient to mediate robust salivary output. Consistent with this concept, pilocarpine (15 mg/kg, s.c.) induced the flow of similar amounts of saliva in M_1/M_4, M_2/M_3, and M_2/M_4 receptor double-KO mice, compared with their corresponding WT control mice (Fig. 7). The observation that a high dose of a muscarinic agonist can induce a maximum secretion response in both the M_1 and M_3 receptor KO mice can be explained best by assuming that M_1 and M_3 receptors are coexpressed by the saliva-producing acinar cells. If M_1 and M_3 receptors were expressed by different subpopulations of acinar cells, one would expect to observe reduced salivation responses in both the M_1 and M_3 receptor single-KO mice, even at maximum agonist doses.

Quantitative real-time RT-PCR experiments (TaqMan) showed that 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 salivary gland tissues (Fig. 2). It is therefore unlikely that the robust salivation responses observed in the M_1 and M_3 receptor single-KO mice after the administration of high doses of pilocarpine are caused by the compensatory overexpression of other mAChR subtypes. However, we cannot completely rule out minor changes in receptor protein expression levels or changes in receptor coupling properties that may occur despite unchanged receptor expression levels.

As discussed above, the pattern of salivation responses obtained with the lowest pilocarpine dose used (1 mg/kg) indicates that the M_3 receptor plays a key role in mediating stimulation of salivary flow in *vivo*, consistent with previous studies (Laniyonu et al., 1990; Dai et al., 1991; Caulfield, 1993; Watson et al., 1996; Moriya et al., 1999; Matsui et al., 2000; Bockman et al., 2001; Bymaster et al., 2003). The predominant functional role of M_3 receptors in stimulating salivary secretion (at least at low agonist concentrations) is most probably a consequence of the preferential expression of M_3 receptors in salivary gland tissue. In the present study, [^{3}H]NMS binding studies indicated that ~65% of the mAChRs present in submandibular gland tissue are M_3 receptors, ~25% are M_1 receptors, and <10% represent other mAChR subtypes (Fig. 6). Quantitative RT-PCR studies indicated that the small population of non-M_1/M_3 mAChRs probably represents a mixture of M_2, M_4, and M_5 receptors (Fig. 2). It should also be noted in this context that immunoprecipitation studies have shown that rat sublingual gland tissue expresses approximately similar numbers of M_1 and M_3 receptors (Watson and Culp, 1994). Moreover, radioligand binding studies suggest that human salivary gland tissues also express a mixture of M_1 and M_3 mAChRs (Giraldo et al., 1988; Mei et al., 1990).

On the other hand, radioligand binding (Hammer et al., 1980; Dai et al., 1991; Bockman et al., 2001), functional (Dai et al., 1991; Bockman et al., 2001), and immunoprecipitation studies (Dai et al., 1991) indicate that the rat parotid gland almost exclusively expresses M_3 receptors. The contribution of M_1 receptors to muscarinic agonist (acetylcholine)-mediated stimulation of salivary secretion (see below) may therefore be mediated predominantly by M_1 receptors present in the submandibular and sublingual glands.

Several recent studies suggest that non-M_1/M_3 mAChRs may also play a role in cholinergic stimulation of glandular secretion. For example, it has been proposed based on studies using subtype-preferring muscarinic antagonists that M_5 receptors may be present/functional in salivary gland tissue (Flynn et al., 1997; Meloy et al., 2001; Tobin et al., 2002). Moreover, Takeuchi et al. (2002) reported that salivary secretion induced by pilocarpine (1 mg/kg, s.c.) was slightly reduced (by ~10–20%) in M_5 receptor KO mice. Finally, Bymaster et al. (2003) recently found that administration of a single dose of oxotremorine (0.3 mg/kg, s.c.) resulted in a reduced salivation response in M_4 receptor single-KO mice (reduction in saliva output by ~35% measured over a 15-min observation period).

To test the hypothesis that non-M_1/M_3 mAChRs play a significant role in cholinergic stimulation of salivary secretion in vivo, we generated and analyzed mice in which both the M_1 and M_3 receptor genes had been inactivated (M_1/M_3 receptor double-KO mice). It was remarkable that pilocarpine, at any of the three doses tested, was no longer able to stimulate salivary secretion in M_1/M_3 receptor double-KO mice (Fig. 3). However, the β-adrenergic receptor agonist (*S*)-isoproterenol (1 mg/kg, s.c.) retained the ability to stimulate saliva production in M_1/M_3 receptor double-KO mice with high efficacy (Fig. 4), indicating that the functional deficits displayed by the M_1/M_3 receptor double-KO mice were receptor-specific and not caused by changes in the expression levels of downstream signaling components. Our observation that pilocarpine-mediated stimulation of salivary flow in vivo is abolished in the M_1/M_3 receptor double-KO mice indicates that non-M_1/M_3 receptors, such as M_4 and M_5 receptors, are unlikely to make a significant contribution to muscarinic agonist-induced saliva production. The functional deficits displayed by the M_4 (Bymaster et al., 2003) and M_5 receptor single-KO mice (Takeuchi et al., 2002) are therefore most probably caused by other factors, such as

mouse genetic background or altered expression levels of other signaling proteins.

Takakura et al. (2003) recently showed that pilocarpine, which readily enters the brain, can stimulate salivary secretion by activating central mAChRs, probably by increasing parasympathetic outflow. Because the M_4 and M_5 mAChRs are preferentially expressed in the CNS (Wess, 1996; Caulfield and Birdsall, 1998), the reduction in pilocarpine- or oxotremorine-induced stimulation of salivary function observed in M_4 (Bymaster et al., 2003) and M_5 (Takeuchi et al., 2002) mAChR KO mice could therefore also be caused by the lack of neuronal rather than glandular M_4 or M_5 receptors.

Although our data suggest that drugs devoid of activity at M_1 and M_3 receptors should lack adverse effects on salivation, clinical studies indicate that relative potency at M_3 mAChRs may not be a reliable predictor of xerostomia. For example, administration of the muscarinic antagonist solifenacin, which is endowed with a moderate degree of M_3 receptor selectivity, seems to cause less pronounced dry-mouth symptoms than tolterodine (nonselective) or darifenacin, another M_3 receptor-preferring antagonist (Ikeda et al., 2002; Chapple et al., 2004a,b; Kobayashi et al., 2004). These differences in drug effects on salivary gland function observed in clinical studies may be caused by differences in pharmacokinetics, drug stability, or other as-yet-unknown parameters that affect drug activity at salivary glands in vivo.

In conclusion, our results indicate that muscarinic agonists trigger salivary secretion in vivo by activating a mixture of glandular M_1 and M_3 mAChRs. Other glandular mAChR subtypes are unlikely to be involved in mediating this activity, at least not in the mouse. These findings should be highly relevant for the development of novel, clinically useful muscarinic drugs.

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