Roles of M$_2$ and M$_4$ muscarinic receptors in regulating acetylcholine release from myenteric neurons of mouse ileum.

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Running head: presynaptic muscarinic receptor in enteric neuron

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Abstract

We investigated the subtype of presynaptic muscarinic receptors associated with inhibition of acetylcholine (ACh) release in the mouse small intestine. We measured endogenous ACh released from longitudinal muscle with myenteric plexus (LMMP) preparations obtained from M₁-M₅ receptor knockout (KO) mice. Electrical field stimulation (EFS) increased ACh release in all LMMP preparations obtained from M₁-M₅ receptor single KO mice. The amounts of ACh released in all preparations were equal to that in the wild-type mice. Atropine further increased EFS-induced ACh release in the wild-type mice. Unexpectedly, atropine also increased, to a similar extent, EFS-induced ACh release to the wild-type mice in all M₁-M₅ receptor single KO mice. In M₂ and M₄ receptor double KO mice, the amount of EFS-induced ACh release was equivalent to an atropine-evoked level in the wild-type mouse, and further addition of atropine had no effect. M₂ receptor-immunoreactivity was located in both smooth muscle cells and enteric neurons. M₄ receptor-immunoreactivity was located in the enteric neurons, being in colocalization with M₂ receptor-immunoreactivity. These results indicate that both M₂ and M₄ receptors mediate the muscarinic autoinhibition in ACh release in the LMMP preparation of the mouse ileum, and loss of one of these subtypes can be compensated functionally by a receptor that remained. Neither M₁, M₃, nor M₅ receptors appear to be involved in this mechanism.

Key words; presynaptic muscarinic receptor; knockout mouse; ileal myenteric plexus; endogenous acetylcholine release; M₂ and M₄ receptors
Introduction

Muscarinic receptors present on nerve terminals play important roles in regulating the release of neurotransmitters (Starke et al. 1989). In particular, it is well known that activation of muscarinic receptors present on cholinergic nerve terminals inhibits acetylcholine (ACh) release in many tissues, this process being called autoinhibition (Somogyi and de Groat 1999; Starke et al. 1989). Although five distinct subtypes of muscarinic receptors were identified (Eglen et al. 1996), the subtype of muscarinic receptors involved in the regulation of the neurotransmitter release has not been clarified in detail. The reasons may be due in part to the lack of muscarinic agonists and antagonists that show a high degree of selectivity for the individual subtype of muscarinic receptors and in part to the multiple expression of the subtypes in most organs, tissues and cells (Wess 2004). Recently, mouse strains deficient in each of the five muscarinic receptor subtypes were generated by employing gene-targeting techniques (Gomeza et al. 1999a; Hamilton et al. 1997; Matsui et al. 2000). Studies using these mice have suggested the subtypes of muscarinic receptor regulating the release of the neurotransmitter. In hippocampal and cortical slices prepared from M\textsubscript{2} receptor-knockout (KO) mice, oxotremorine did not affect $[^3]$H\textsubscript{ACh} release induced by a high K$^+$ concentration, although it inhibited the release in those slices prepared from wild-type mice (Zhang et al. 2002a). In contrast, oxotremorine did not affect the release in the striatal slice prepared from M\textsubscript{4} receptor KO mice (Zhang et al. 2002a). Also, in peripheral tissues, it was suggested that ACh release from the urinary bladder and atria was regulated to a large and to a less extent by M\textsubscript{4} receptors (Zhou et al. 2002), whereas release from the diaphragm was regulated by M\textsubscript{2} receptors (Slutsky et al. 2003). These results suggest that the subtype of muscarinic receptors modulating ACh release differs among the organs and tissues. The heterogeneity of muscarinic receptors modulating the neurotransmitter release was also suggested by results obtained in the sympathetic nervous system (Trendelenburg et al. 2003). In the gastrointestinal tract, activation of presynaptic muscarinic receptors resulted in inhibition of ACh release from enteric neurons in the guinea pig ileum (Kilbinger and Wessler 1980) and stomach (Ogishima et al. 2000), and in the rat
ileum (Coulson et al. 2002). However, the subtype of muscarinic receptors that participates in autoinhibition has not been examined in detail in the mouse. Studies on autoinhibition in the mouse intestine provide an unique advantage because KO mice deficient in each subtype of the muscarinic receptor enable us to better assess the role of the individual subtypes in regulating ACh release from the myenteric neurons. In fact, studies using muscarinic receptor KO mice, as noted above, have clarified the subtype of the muscarinic receptor in tissues other than the intestine. In the present study, we investigated the subtype of muscarinic receptors involved in the autoinhibition of ACh release by examining ACh released endogenously from myenteric neurons of muscarinic receptor KO mice, M₁-M₅ receptor KO mice, and M₂/M₃ and M₂/M₄ receptor double KO mice.
Materials and Methods

Animal

The generation of homozygous M1-M5 receptor KO mice, and M2/M3 and M2/M4 receptors double KO mice has been described previously (Fukudome et al. 2004; Karasawa et al. 2003; Matsui et al. 2002; Matsui et al. 2000; Nakamura et al. 2004; Ohno-Shosaku et al. 2003). KO mice were backcrossed with C57BL/6 mice for 8-10 generations. Age-matched wild-type C57BL/6 mice were included as controls. Mouse genotyping was carried out by PCR analysis of mouse-tail DNA. Adult (2-6 months old) mice were used in this study. Animal maintenance and experimental procedures were performed in accordance with the guidelines of the ethics committees of Osaka Prefecture University and the Institute of Medical Science, the University of Tokyo.

Release of acetylcholine

Mice of either sex, weighing 18-30 g, were lightly anesthetized with diethyl ether and sacrificed by bleeding. The longitudinal muscle preparations of the mouse ileum including the myenteric plexus were made using a method for guinea pig ileum described previously (Takeuchi et al. 2001). The preparation was mounted in an organ bath containing 3 ml of Tyrode solution of the following composition: 136.9 mM NaCl, 2.7 mM KCl, 1.8 mM CaCl2, 1.05 mM MgCl2, 11.9 mM NaHCO3, 0.4 mM NaH2PO4 and 5.6 mM glucose. A bathing medium was kept at 37°C and bubbled with 95% O2 and 5% CO2.

The preparations were equilibrated for 30 min by perfusion with Tyrode solution containing physostigmine salicylate (5 µM) and choline chloride (1 µM) at a rate of 1-2 ml min⁻¹. Then, perfusion was stopped and the bathing medium was replaced by 3 ml of fresh Tyrode solution at intervals of 4 min. After two consecutive samples were collected for the measurement of spontaneous release of ACh, the preparations were stimulated by electrical field stimulation (EFS). For the EFS-induced release of ACh, the stimulation was performed at supramaximal voltage (50 V), pulse duration of 0.5 msec, 600 pulses at 10 Hz (Fig. 1). Bathing fluid was collected after a further 180-sec period. These experimental procedures
were repeated twice with 15 min intervals between sample collection. EFS was carried out with a pair of platinum electrodes, one at the top and the other at the bottom of the preparation. The first stimulation (S₁) was carried out in the absence of test drug(s), and the second (S₂) was in the presence of test drug(s). All samples collected were kept on ice until the end of the ACh release experiment, and then processed for ACh determination. At the end of the experiment, the preparations were blotted and weighed. Acetylcholine release due to EFS (S₁', S₂') was calculated by subtracting the output of the immediately preceding spontaneous release (R₁, R₂) from the total output during the periods of stimulation. Part of results are shown as relative ACh release defined as R₂/R₁ x 100 and S₂'/S₁' x 100. Muscarinic antagonists were treated 15 min before the second collection period.

ACh released in the medium was assayed by HPLC, using a postcolumn enzymes (acetylcholinesterase plus choline oxidase) reactor (Eicom AC-Enzympak; Eicom, Kyoto, Japan) as described elsewhere (Takeuchi et al. 2001).

**PCR amplification of M₂ and M₄ receptor cDNA and RT-PCR analysis of expression of muscarinic receptors in mouse ileum**

Total RNA from ileum of wild-type, M₂ receptor KO and M₄ receptor KO mouse was isolated with SV Total RNA Isolation System containing DNase I (Promega WI, USA) and reverse-transcribed with random hexamers primer using SuperScript First-Strand synthesis System for RT-PCR (Invitrogen San Diego, USA), according to the manufacturer’s instructions. The cDNAs of coding regions of M₂ and M₄ receptors were amplified by PCR with Taq polymerase (Takara, Tokyo, Japan). For analysis of expression of M₂ and M₄ receptors in mouse ileum, we designed specific primers of each receptor. Primer sequences are indicated as follows: for mouse M₂ receptor (AF264049), M₂F (5'-CAG CCA GAC TCC ACC AGA TC-3') and M₂R (5'-CCA ATC ACA GTG TAG AGG GT-3'); and for mouse M₄ receptor (X63473), M₄F (5'-AGC TTT GAC CGC TA T TTC TGC GTC-3') and M₄R (5'-CAT CAG AGG GCT CTT GAG GAA AGC-3'). PCR amplification was performed for 30 cycles at 94°C for 1 min, at 55°C for 1 min, and then 72°C for 1 min followed by 72°C for 8 min.
Amplified DNA fragments were separated on a 2% agarose gel.

**Antibody**

Rat monoclonal antisera against M<sub>2</sub> muscarinic receptor (MAB367), goat polyclonal antisera against choline acetyltransferase (ChAT; AB144P) and rabbit polyclonal antisera against neurofilament 150 kDa (AB1981) were purchased from Chemicon International (Temecula, CA, USA). Rabbit polyclonal antibody against M<sub>4</sub> muscarinic receptor (H-175) was purchased from Santa Cruz biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal antisera against synaptophysin (08-1130) and glial fibrillary acidic protein (GFAP; clone 6F-2) were purchased from Zymed laboratories (San Francisco, CA, USA) and DAKO JAPAN (Kyoto, Japan), respectively.

**Immunohistochemical study**

Immunohistochemical study was carried out by the method described previously (Fujita et al. 2003). Briefly, the intestine was isolated after the mice were deeply anesthetized with pentobarbital sodium (50 mg/kg<sup>-1</sup>, i.p.) and the intestine was fixed by transcardiac perfusion. The intestine was dissected, postfixed with 4% (w/v) paraformaldehyde in 0.1 M phosphate buffer (PB solution, pH 7.4) for 24 hr, dehydrated with 30% (w/v) sucrose solution, and then frozen with O.C.T. (Optimal Cutting Slice Temperature) compound (Tissue-Tek, Sakura Finetechnology Inc., Tokyo). For whole-mount preparations, short segments of the intestine were inflated and the mucosa was removed with a small razor, and the remaining strips (5 x 5 mm) were pinned to the silicon rubber. The tissues were fixed for 2 hr at room temperature with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Following fixation, sectional and whole-mount preparations were washed three times with phosphate-buffered saline (PBS) and then placed in PBS containing 0.5% Triton X-100, 1% bovine serum albumin, and 10% normal goat or donkey serum for 1 hr at room temperature to avoid nonspecific staining. The preparations were then incubated with anti-M<sub>2</sub> receptor (1:200), anti-M<sub>4</sub> receptor (1:200), anti-synaptophysin (1:1000), anti-neurofilament (1:1000), anti-
ChAT (1:1000) or anti-GFAP (1:1000) antibodies in PBS at 4°C for 24 hr. Immunoreactivity of each of the antibody was detected using Alexa flour 568-conjugated anti-rat IgG (Molecular Probes, Eugene, OR, USA), fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG or anti-goat IgG (Jackson Immuno Research Laboratories, West Grove, PA, USA) secondary antibodies. Confocal images were obtained under a laser scanning microscope (MRC-1024; Bio-Rad, Hertfordshire, UK).

Statistical analysis

All values were expressed as mean ± SEM. The differences between the values were evaluated by ANOVA, and thereafter assessed by either Student’s t-test or Welch test (if significant differences were indicated by ANOVA). P values < 0.05 were considered significant.

Drugs

Choline chloride, atropine sulphate, tetrodotoxin and physostigmine salicylate were purchased from Wako Pure Chemical (Osaka, Japan). All other chemicals were of analytical grade.
Results

In wild-type mouse ileum, the amount of ACh released spontaneously from the LMMP preparations was 441 ± 45 pmol/g tissue/min. Electrical filed stimulation (EFS) at 10 Hz increased the ACh release to 6425 ± 741 pmol/g tissue/min (Table 1). In control experiments, no significant difference was observed between the first (S_1') and the second (S_2') stimulation in terms of the release of ACh induced by EFS. The value for the relative amounts of released ACh (S_2'/ S_1') was 111.9 ± 8.6 % (n = 5). Atropine (1 µM), which maximally enhanced EFS-induced ACh release from the myenteric neurons of the guinea pig ileum (Saitoh et al. 1997), increased EFS-induced ACh release to about 1.8 times that of the control (in the absence of atropine), but did not increase the spontaneous release (Fig. 2). Tetrodotoxin (TTX), at 1 µM, completely inhibited EFS-induced ACh release (S_2'/ S_1' = 4.3 ± 1.6 %, n = 3), whereas it only slightly inhibited the spontaneous release (R_2/R_1 = 83.3 ± 9.1 %, n = 3).

We next examined ACh release from the LMMP preparations obtained from the muscarinic receptor KO mice. There was no significant difference among the values of the spontaneous ACh release in M1-M5 receptor KO and wild-type mice (Table 1). As shown in Table 1, EFS-induced ACh release was also not significantly different among M1-M5 receptor KO and wild-type mice. The relative values (S_2'/ S_1') of EFS-induced release in each of M1-M5 receptor KO mice were 113.5 ± 3.6 (n = 5), 91.5 ± 10.4 (n = 6), 105.7 ± 11.8 (n = 4), 99.1 ± 7.1 (n = 6) and 98.3 ± 6.2 % (n = 4) for M1, M2, M3, M4 and M5 receptor KO mice, respectively. Atropine (1 µM) increased EFS-induced ACh release from M1-M5 receptor KO mice to a similar extent as that observed in the wild-type mice (Fig. 3). Thus, lack of muscarinic receptor subtypes did not affect the spontaneous and EFS-induced ACh release and the effects of atropine (Table 1).

We then studied the release of ACh from the LMMP preparations obtained from the muscarinic receptor double KO mice. Since it was previously shown that the M3 and M4 receptors regulated ACh release in the central nervous system (Zhang et al. 2002a), we first used the preparation obtained from M2 and M4 (M2/M4) receptor double KO mice. The
amount of the EFS-induced ACh release in the M_2/M_4 receptor double KO mice was significantly greater than that in the wild-type mice, whereas the spontaneous release remained unchanged (Table 2). The relative value (S'_2 / S'_1) of EFS-induced ACh release in M_2/M_4 receptor double KO mice was 100.5 ± 7.9 % (n = 5). TTX completely inhibited the EFS-induced release (S'_2 / S'_1 = 0.4 ± 0.3 %, n = 3). Interestingly, atropine (1 µM) did not further increase the EFS-induced release (Fig 4). In other words, EFS-induced ACh release had been increased to the maximum level prior to the presence of atropine in this double KO mouse. On the other hand, in M_2/M_3 receptor double KO mice, the amounts of spontaneous and EFS-induced ACh release, and the effect of atropine were roughly equal to those in wild-type mice (Table 2, Fig. 4). Thus, it seems likely that the presence of M_2 and M_4 receptors participates in the increase in ACh release in the presence of atropine.

To examine whether M_2 and M_4 receptors are expressed in the mouse ileum, RT-PCR analysis of total RNA prepared from the ileum of wild-type, M_2 and M_4 receptor KO mice was performed using specific primers. In the wild-type mouse ileum, the expression of M_2 and M_4 receptors was confirmed (Fig. 5). The expression of M_4 and M_2 receptor was confirmed in the ileum of M_2 and M_4 receptor KO mouse, respectively (Fig. 5).

We next investigated the localization of the M_2 and M_4 muscarinic receptors in the mouse ileum by immunohistochemistry using a monoclonal rat IgG antibody for the mouse M_2 receptor and a polyclonal rabbit IgG antibody for the M_4 receptors. The strong immunoreactivity of an anti-M_2 receptor antibody was observed in the plasma membrane of smooth muscle cells within ileal circular and longitudinal muscle layers, and myenteric plexus in the wild-type mouse ileum (Fig 6A, B). We then stained the mouse ileal tissue with antibodies which recognize neurons and glial cells. Immunoreactivities of anti-neurofilament and anti-synaptophysin antibodies, markers of neurons, were located in the myenteric plexus. The localization was observed in parallel with smooth muscle cells in the circular muscular layer (Fig. 6B). Double staining with an anti-M_2 receptor antibody and these neuron markers produced a prominent yellow signal in the myenteric plexus and muscular layers (Fig. 6A, B), indicating the localization of M_2 receptors in neurons. In
contrast, an immunoreactivity of M₂ receptors was not colocalized with that of anti-GFAP antibody (Fig. 6A), a marker of glial cells, as described previously (Fujita et al. 2001). A large part of the immunoreactive site to anti-M₂ receptor antibody was also stained with an anti-choline acetyltransferase (ChAT) antibody, a marker of the cholinergic neuron (Fig. 6C). To examine the specificity of anti-M₂ receptor antibody, the ileal preparation of M₂ receptor KO mouse was stained with anti-M₂ and anti-M₄ receptor antibodies. Immunoreactivity of anti-M₂ receptor antibody was not observed in the myenteric plexus, while that of anti-M₄ receptor antibody was observed (Fig. 6D). Immunoreactivity of the anti-M₄ receptor antibody was localized in the myenteric plexus similar to that of anti-M₂ receptor antibody (Fig. 7A). However, although smooth muscle cells were not stained with anti-M₄ receptor antibody, immunoactive sites to the M₄ receptor were observed in muscular layers (Fig. 7A). Double staining with anti-M₄ receptor and anti-synaptophysin antibodies produced a yellow signal in the myenteric plexus and muscular layers (Fig. 7A), indicating the localization of M₄ receptors in neurons. A large part of the immunoreactive site to M₄ receptors was also stained with an anti-M₂ receptor antibody (Fig. 7B), indicating the colocalization of both receptors in myenteric neurons. In the myenteric plexus of M₄ receptor KO mouse, immunoreactivity of anti-M₄ antibody was scarcely observed (Fig. 7C).
Discussion

There are many reports indicating the involvement of presynaptic muscarinic receptors in the regulation of ACh release from the myenteric neurons in many animal species with exception of the mouse (Kilbinger et al. 1993). Although five subtypes of muscarinic receptors have been cloned, the subtype of the muscarinic receptor involved in the regulation of ACh release is unclear. In the present study, we examined ACh release from LMMP preparations of the mouse ileum and found that ACh release was regulated by presynaptic muscarinic receptors in a similar manner to that in other animal species (Saitoh et al. 1997): atropine did not affect the spontaneous ACh release, but significantly increased the EFS-induced release (Fig. 2). Mutant mouse lines deficient in an individual gene of the five muscarinic receptor subtypes have recently become available (Matsui et al. 2004; Wess 2004). We, therefore, aimed to identify the subtype of the presynaptic muscarinic receptor that regulates ACh release by using muscarinic receptor KO mice.

The amounts of ACh released from the LMMP preparations of M1-M5 receptor single KO mice were similar to that observed in the wild-type mouse. Atropine increased EFS-induced release to the same extent in the wild-type and all single KO mice. These results suggest that presynaptic inhibition by the muscarinic receptors are not mediated by one subtype, although small association of single receptor subtype with the inhibition could not be necessarily completely excluded, due to a reason of relatively large deviation of the results. Therefore, we further studied ACh release in the muscarinic receptor double KO mice. In the LMMP preparation of M2/M4 receptor double KO mouse, EFS-induced ACh release was significantly increased. The stimulatory effect of atropine was not observed in the double KO mouse. The absolute amount of EFS-induced ACh release in the M2/M4 receptor double KO mouse was roughly equal to that of the wild-type mouse in the presence of atropine. Namely, EFS-induced ACh release in the M2/M4 double KO mice had been increased regardless of atropine treatment. Such an increase was not shown in M2 and M4 receptor single KO and M2/M3 receptor double KO mice, indicating importance of both M2 and M4 receptors for the muscarinic autoinhibition mechanism. The regulation of release of
neurotransmitter with two or more muscarinic receptors has been evident in the hippocampus (Tzavara et al. 2003), striatum (Zhang et al. 2002b), atria (Zhou et al. 2002) and atria and bladder (Trendelenburg et al. 2003) of mouse.

It has been previously shown that a deficiency in either M₂ or M₄ receptor results in the dysregulation of acetylcholine release in the hippocampus (Tzavara et al. 2003). However, the situation in mouse ileum may differ from that in the hippocampus. The amount of ACh release induced by EFS and the effect of atropine did not change in either M₂ or M₄ receptor single KO mouse, compared with wild-type mouse. The finding of RT-PCR revealed the expression of M₂ and M₄ receptors in the ileum of wild-type mouse. Further, it was confirmed to be devoid of M₂ and M₄ receptors in M₂ and M₄ receptor single KO mouse, respectively. When both receptors were absent, EFS-induced ACh release was increased and became resistant to treatment with atropine. These results suggest that activation of a single muscarinic receptor subtype is sufficient to maximally activate autoinhibition. The reason for the difference in a compensatory adaptation to the loss of M₂ or M₄ muscarinic receptors between the enteric and central neurons remains unknown and needs to be further investigated. In the present study, it is unclear how the function of the lost receptor was compensated. Several studies showed that disruption of one specific muscarinic receptor gene does not have major effects on the levels of expression of the remaining four muscarinic receptors (Gomeza et al. 1999a; Gomeza et al. 1999b; Yamada et al. 2001). If this is also the case in mouse enteric neurons, the function of the remaining receptors for modulating ACh release may be potentiated.

Localization of M₁-M₄ receptors has been demonstrated in the rabbit ileum (Levey 1993). Using a competitive binding assay, it was also shown that M₂ receptors were expressed in smooth muscle cells of the rat and guinea pig ileum (Giraldo et al. 1987). In the present study, strong immunoreactivity of the M₂ receptor was observed on the plasma membrane of smooth muscle cells in longitudinal and circular layers of the wild-type mouse. Interestingly, myenteric ganglia are also positive to an anti-M₂ receptor antibody. Glial and neuronal cells are the main components within the myenteric plexus (Fujita et al. 2001). The
cells within myenteric ganglia immunoreactive to anti-M₂ receptor antibody were not stained with anti-GFAP antibody, but stained with anti-synaptophysin and anti-neurofilament antibodies. A part of the circular muscle layer that was neuronal markers-positive was also immunoreactive to anti-M₂ receptor antibody. These results demonstrate that M₂ receptors are expressed in some enteric neurons. Furthermore, the result obtained with an anti-ChAT antibody indicates that these neurons are cholinergic neurons. This observation is consistent with the report that M₂ receptors were expressed in cholinergic nerve terminals in the mouse striatum (Zhang et al. 2002a). On the other hand, M₄ receptors were located in myenteric plexus and muscle layers, but not in smooth muscle cells. Results in the M₄ receptor and synaptophysin double staining support the idea that M₄ receptors are expressed in enteric neurons. In the central nervous system, M₄ receptors are known to be expressed in cholinergic neurons (Hersch et al. 1994; Zhang et al. 2002a). Furthermore, substantial members of M₄ receptor-immunopositive cells were positive to immunoreactivity of anti-M₂ antibody. In the present study, colocalization of M₂ and M₄ receptors was shown in the myenteric cholinergic neurons of the mouse.

The presynaptic muscarinic receptors have been suggested to be M₁ receptors from studies with various muscarinic antagonists in the guinea pig ileum (Dietrich and Kilbinger 1995; Kawashima et al. 1990). However, there is a report that activation of M₁ receptors enhanced the ACh release and activation of presynaptic M₃ subtype inhibited the ACh release in the same guinea pig ileum preparation (Soejima et al. 1993). It was recently suggested that M₂ receptors play an inhibitory role in the ACh release in the rat ileum and guinea pig stomach (Coulson et al. 2002; Ogishima et al. 2000). The reason of these discrepancies may be due to the specificity of antagonists used in these studies, in addition to the difference in species and tissues examined.

The difference in spontaneous release of ACh was not detected among mice used in the present study. In wild-type mouse, atropine did not affect spontaneous release of ACh. The same result was reported in the guinea pig ileum (Nishiwaki et al. 2000). These results suggest that the presynaptic muscarinic receptors are not activated by ACh released
spontaneously. Therefore, the role of muscarinic receptors located in cholinergic terminals may be important when enteric neurons are activated.

It was concluded from the studies described above that a deficiency in M₂ and M₄ receptors resulted in the loss of the muscarinic autoinhibition in ACh release in the mouse small intestine. The results strongly suggest the essential role of these receptors in autoinhibition. Furthermore, it appears that the loss of either receptor was functionally compensated by a remaining receptor.
Acknowledgements

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Table 1  Spontaneous and EFS-induced ACh release from LMMP preparations obtained from the wild-type and muscarinic receptor KO mouse ileum.

<table>
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<th></th>
<th>n</th>
<th>Spontaneous</th>
<th>EFS</th>
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<tr>
<td>Wild</td>
<td>11</td>
<td>441 ± 45</td>
<td>6425 ± 741</td>
</tr>
<tr>
<td>M1-KO</td>
<td>10</td>
<td>537 ± 94</td>
<td>5341 ± 756</td>
</tr>
<tr>
<td>M2-KO</td>
<td>13</td>
<td>544 ± 78</td>
<td>6000 ± 533</td>
</tr>
<tr>
<td>M3-KO</td>
<td>8</td>
<td>463 ± 62</td>
<td>5807 ± 690</td>
</tr>
<tr>
<td>M4-KO</td>
<td>13</td>
<td>488 ± 48</td>
<td>6271 ± 572</td>
</tr>
<tr>
<td>M5-KO</td>
<td>8</td>
<td>370 ± 47</td>
<td>6216 ± 616</td>
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Values are expressed as the mean ± S.E.M. For further details, see Fig. 1 and the Methods.
Table 2  Spontaneous and EFS-induced ACh release from LMMP preparations obtained from M₂ and M₄ (M₂/M₄), and M₂ and M₃ (M₂/M₃) KO mouse ileum.

<table>
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<th></th>
<th>n</th>
<th>Spontaneous</th>
<th>EFS</th>
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<tbody>
<tr>
<td>M₂/M₄-KO</td>
<td>11</td>
<td>433 ± 56</td>
<td>10961 ±1200**</td>
</tr>
<tr>
<td>M₂/M₃-KO</td>
<td>10</td>
<td>311 ± 41</td>
<td>7296 ± 636</td>
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Values are expressed as the mean ± S.E.M.
Figure legends

Figure 1. Protocol for the experiment on acetylcholine (ACh) release. The spontaneous and the EFS-induced release of ACh was measured in the presence and absence of test drug(s). Black rectangles indicate the duration (60 sec) of EFS. For further details, see Materials and Methods.

Figure 2. Effects of atropine on spontaneous and EFS-induced release of ACh from longitudinal muscle with myenteric plexus (LMMP) preparations of wild-type mouse ileum. Atropine was applied 15 min before the second trial. Values are the mean ± S.E.M for 6 independent experiments. Spontaneous and EFS (10 Hz)-induced release was expressed as the net amount of ACh release. *Significantly different from the value in the absence of atropine at P<0.05 (Student’s t-test). For further details, see the Materials and Methods.

Figure 3. Effects of atropine on EFS-induced ACh release from LMMP preparations of muscarinic receptor single KO mouse ileum. In M1-M5 receptor KO mice, ACh release was evoked by EFS at 10 Hz in the presence or absence of 1 µM atropine. Atropine was applied 15 min before the second trial. Values are the mean ± S.E.M for 4-6 independent experiments. Evoked release was expressed as the net amount of ACh released by EFS. *Significantly different from the value in the absence of atropine at P<0.05 (Student’s t-test). For further details, see the Materials and Methods.

Figure 4. Effects of atropine on EFS-induced ACh release from LMMP preparations of muscarinic receptor double KO mouse ileum. In M2/M4 and M2/M3 receptor double KO mice, ACh release was evoked by EFS at 10 Hz in the presence or absence of 1 µM atropine. Atropine was applied 15 min before the second trial. Values are the mean ± S.E.M for 5-6 independent experiments. Evoked release was expressed as the net amount of ACh release by EFS. *Significantly different from the value in the absence of atropine at P<0.05 (Student’s t-test). For further details, see the Materials and Methods.

Figure 5. RT-PCR analysis of M2 and M4 muscarinic receptor expression in mouse ileum.
Representative 2% agarose gels (stained with ethidium bromide) are shown. Primers specific for the individual subtype of mouse muscarinic receptors were used to amplify cDNA prepared from total RNA of the mouse ileum. M₂ and M₄ receptors were expressed in the wild-type mouse ileum. M₂ and M₄ receptors were not present in M₂ receptor KO and M₄ receptor KO mouse, respectively. As a positive control, expression of β-actin mRNA was shown. Numbers on the left indicate the size markers in base pairs (bp). Three separate experiments gave similar results.

Figure 6. Localization of M₂ receptors in the ileum of wild-type and M₂ receptor KO mice.

Immunofluorescence of M₂ receptor was detected by a confocal microscopy. The tissue sections (A) and whole-mount preparations (B, C and D) were treated with the anti-M₂ receptor antibody and stained with Alexa Flour 568-conjugated anti-rat IgG antibody (red). Colocalization is visualized as yellow in the merged images. A) Images of double staining with anti-M₂ receptor antibody, and anti-neurofilament, -synaptophysin and -GFAP (green) antibodies in the wild-type mouse ileum. B) Images of double staining with anti-M₂ receptor and anti-synaptophysin antibodies in the circular muscle (CM) and myenteric plexus layers (MP). Some synaptophysin-immunopositive sites were also stained with anti-M₂ receptor antibody (arrowheads in CM). C) Images of double staining with anti-M₂ receptor and anti-choline acetyltransferase (ChAT) antibodies in MP. D) Images of anti-M₂ and anti-M₄ receptor antibodies in MP of M₂ receptor KO mouse. Scale bars, 50 µM.

Figure 7. Localization of M₄ receptors in the ileum of wild-type and M₄ receptor KO mice.

The whole-mount preparations were treated with the anti-M₄ receptor antibody and stained with FITC-conjugated anti-rabbit IgG antibody. Colocalization is visualized as yellow in the merged images. A) Images of double staining with anti-M₄ receptor and anti-synaptophysin antibodies in the circular muscle (CM) and myenteric plexus layers (MP). Some synaptophysin-immunopositive sites were also stained with anti-M₄ receptor antibody. B) Images of double staining with anti-M₂ and anti-M₄ receptor antibodies in MP. Note that all M₄-immunopositive cells were stained with anti-M₂ receptor antibody. C) An image of anti-M₄ receptor antibody in MP of M₄ receptor KO mouse. Scale bars, 50 µM.
Takeuchi-Figure 1

Resting perfusion

Spontaneous ($R_1$) | Evoked ($S_1$) | Drug treatment

Resting perfusion

Spontaneous ($R_2$) | Evoked ($S_2$)

EFS 60 S

0

30

34

38

53

57

61 min

