Roles of M2 and M4 Muscarinic Receptors in Regulating Acetylcholine Release From Myenteric Neurons of Mouse Ileum

Tadayoshi Takeuchi,1 Kaori Fujinami,1 Hiroto Goto,1 Akikazu Fujita,1 Makoto M. Taketo,2 Toshiya Manabe,3 Minoru Matsui,3 and Fumiaki Hata1

1Department of Veterinary Pharmacology, Graduate School of Agriculture and Life Science, Osaka Prefecture University, Sakai Osaka; 2Department of Pharmacology, Graduate School of Medicine, Kyoto University, Kyoto; and 3Division of Neuronal Network, Department of Basic Medical Sciences, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan

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Takeuchi, Tadayoshi, Kaori Fujinami, Hiroto Goto, Akikazu Fujita, Makoto M. Taketo, Toshiya Manabe, Minoru Matsui, and Fumiaki Hata. Roles of M2 and M4 muscarinic receptors in regulating acetylcholine release from myenteric neurons of mouse ileum. J Neurophysiol 93: 2841–2848, 2005; doi:10.1152/jn.00986.2004. 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 M2–M5 receptor knockout (KO) mice. Electrical field stimulation (EFS) increased ACh release in all LMMP preparations obtained from M2–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 M2–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 co-localization with M4 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. M1, M3, and M4 receptors do not seem to be involved in this mechanism.

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 (Gomez 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 M2 receptor knockout (KO) mice, oxotremorine did not affect [3H]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 M4 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 M4 receptors (Zhou et al. 2002), whereas release from the diaphragm was regulated by M2 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 this 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, M2–M5 receptor KO mice, M2/M3 receptor double KO mice, and M2/M4 receptor double KO mice.

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Address for reprint requests and other correspondence: T. Takeuchi, Dept. of Veterinary Pharmacology, Graduate School of Agriculture and Life Science, Osaka Prefecture Univ., 1-1 Gakuen-cho, Sakai Osaka 599-8531, Japan (E-mail: Takeuchi@vet.osakafu-u.ac.jp).

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METHODS

Animals

The generation of homozygous M1–M4 receptor KO mice and M1/M3 and M2/M4 receptors double KO mice has been described previously (Fukudome et al. 2004; Karasawa et al. 2003; Matsui et al. 2000, 2002; 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 mo 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 ACh

Mice of either sex, weighing 18–30 g, were lightly anesthetized with diethyl ether and killed by bleeding. The longitudinal muscle preparations of the mouse ileum including the myenteric plexus (LMMP) 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 (in mM): 136.9 NaCl, 2.7 KCl, 1.8 CaCl2, 1.05 MgCl2, 11.9 NaHCO3, 0.4 NaH2PO4 and 5.6 glucose. A bathing medium was kept at 37°C and bubbled with 95% O2-5% CO2.

The preparations were equilibrated for 30 min by perfusion with Tyrode solution containing phystostigmine salicylate (5 μM) and choline chloride (1 μM) at a rate of 1–2 ml/min. 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 ms, 600 pulses at 1,000 Hz. After each stimulation, the preparations were washed three times with Tyrode solution and the medium was replaced by 3 ml of fresh Tyrode solution. 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 experiment, the preparations were blotted and weighed. Part of release of ACh, the stimulation was performed at 11.9 NaHCO3, 0.4 NaH2PO4 and 5.6 glucose. A bathing medium was kept at 37°C and bubbled with 95% O2-5% CO2.

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 mice was isolated with SV Total RNA Isolation System containing DNase I (Promega) and reverse-transcribed with random hexamers primer using Superscript First-Strand Synthesis System for RT-PCR (Invitrogen), according to the manufacturer’s instructions. The cDNA of coding regions of M2 and M4 receptors was amplified by PCR with Taq polymerase (Takara). 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’-CAGCCAGACTCCACCAGATC-3’) and M2R (5’-CCATCAGTCGAGGT-3’); and for mouse M4 receptor (X63473), M4F (5’-AGCTTTGACCGTATTTCTGCTC-3’) and M4R (5’-CATCAGGGCTTCTGAGGAACC-3’). PCR amplification was performed for 30 cycles at 94°C for 1 min, at 55°C for 1 min, and at 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 M2 muscarinic receptor (MAB367), goat polyclonal antisera against choline acetyltransferase (ChAT; AB144P), and rabbit polyclonal antisera against neurofilament 150 kDa (AB1851) were purchased from Chemicon International (Temecula). Rabbit polyclonal antibody against M2 muscarinic receptor (H-175) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal antisera against synaptophysin (08–1130) and glial fibrillary acidic protein (GFAP; clone 6F-2) were purchased from Zymed laboratories (San Francisco, CA) 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, ip), and the intestine was fixed by transcardiac perfusion. The intestine was dissected, postfixed with 4% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer (PB solution, pH 7.4) for 24 h, dehydrated with 30% (wt/vol) sucrose solution, and frozen with O.C.T. (Optimal Cutting Slice Temperature) compound (Tissue-Tek, Sakura Finetechnology, Tokyo, Japan). For whole-mount preparations, short segments of the intestine were inflated, the mucosa was removed with a small razor, and the remaining strips (5 × 5 mm) were pinned to the silicon rubber. The tissues were fixed for 2 h at room temperature with 4% paraformaldehyde in 0.1 M PB (pH 7.4). After fixation, sectional and whole-mount preparations were washed three times with PBS (0.05 M, pH 7.4) and then incubated in 1% hydrogen peroxide in 0.1 M PB for 30 min. The tissues were washed in 0.1 M PB and then incubated in 0.3% Triton X-100 in 0.1 M PB for 2 h. The tissues were washed in 0.1 M PB and then incubated in 1% bovine serum albumin (BSA) solution in 0.1 M PB for 1 h. The tissues were washed in 0.1 M PB and then incubated with antisera in 1% BSA solution in 0.1 M PB for 2 h at room temperature. After immunohistochemical study, the tissues were washed in 0.1 M PB and then incubated with secondary antibodies in 1% BSA solution in 0.1 M PB for 2 h at room temperature. After immunohistochemical study, the tissues were washed in 0.1 M PB and then incubated with secondary antibodies in 1% BSA solution in 0.1 M PB for 2 h at room temperature. After immunohistochemical study, the tissues were washed in 0.1 M PB and then incubated with secondary antibodies in 1% BSA solution in 0.1 M PB for 2 h at room temperature. After immunohistochemical study, the tissues were washed in 0.1 M PB and then incubated with secondary antibodies in 1% BSA solution in 0.1 M PB for 2 h at room temperature.
times with PBS and placed in PBS containing 0.5% Triton X-100, 1% bovine serum albumin, and 10% normal goat or donkey serum for 1 h at room temperature to avoid nonspecific staining. The preparations were incubated with anti-M2 receptor (1:200), anti-M3 receptor (1:200), anti-synaptophysin (1:100), anti-neurofilament (1:1000), anti-ChAT (1:1000), or anti-GFAP (1:1000) antibodies in PBS at 4°C for 24 h. Immunoreactivity of each of the antibody was detected using Alexa flour 568-conjugated anti-rat IgG (Molecular Probes, Eugene, OR), fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG, or anti-goat IgG (Jackson Immuno Research Laboratories, West Grove, PA) secondary antibodies. Confocal images were obtained under a laser scanning microscope (MRC-1024, Bio-Rad, Hertfordshire, UK).

### Statistical analysis

All values were expressed as means ± SE. 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 < 0.05 was considered significant.

### Drugs

Choline chloride, atropine sulfate, TTX, 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. 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 (S1) and the second (S2) stimulation in terms of the release of ACh induced by EFS. The value for the relative amounts of released ACh (S2/S1) 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). TTX, at 1 μM, completely inhibited EFS-induced ACh release (S2/S1 = 4.3 ± 1.6%, n = 3), whereas it only slightly inhibited the spontaneous release (R2/R1 = 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 and EFS-induced 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 (S2/S1) 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–M3 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 M2 and M4 receptors regulated ACh release in the CNS (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 M2/M4 receptor double KO mice

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

<table>
<thead>
<tr>
<th>Amount of Released ACh, pmol/g tissue/min</th>
<th>n</th>
<th>Spontaneous</th>
<th>EFS</th>
</tr>
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<tbody>
<tr>
<td>Wild</td>
<td>11</td>
<td>441 ± 45</td>
<td>6,425 ± 741</td>
</tr>
<tr>
<td>M1-KO</td>
<td>10</td>
<td>537 ± 94</td>
<td>5,341 ± 756</td>
</tr>
<tr>
<td>M2-KO</td>
<td>13</td>
<td>544 ± 78</td>
<td>6,000 ± 533</td>
</tr>
<tr>
<td>M3-KO</td>
<td>8</td>
<td>463 ± 62</td>
<td>5,807 ± 690</td>
</tr>
<tr>
<td>M4-KO</td>
<td>13</td>
<td>488 ± 48</td>
<td>6,271 ± 572</td>
</tr>
<tr>
<td>M5-KO</td>
<td>8</td>
<td>370 ± 47</td>
<td>6,216 ± 616</td>
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</table>

Values are expressed as mean ± SE. EFS, electrical field stimulation; ACh, acetylcholine; LMMP, longitudinal muscle with myenteric plexus; KO, knockout. For further details, see Fig. 1 and METHODS.

![FIG. 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 2nd trial. Values are means ± SE 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).](http://jn.physiology.org/)

![FIG. 3. Effects of atropine on EFS-induced ACh release from LMMP preparations of muscarinic receptor single knockout (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 2nd trial. Values are means ± SE 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).](http://jn.physiology.org/)
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 M2/M4 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 before the presence of atropine in this double KO mouse. On the other hand, in M2/M3 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 M2 and M4 receptors participates in the increase in ACh release in the presence of atropine.

To examine whether M2 and M4 receptors are expressed in the mouse ileum, RT-PCR analysis of total RNA prepared from the ileum of wild-type and M2 and M4 receptor KO mice was performed using specific primers. In the wild-type mouse ileum, the expression of M2 and M4 receptors was confirmed (Fig. 5). The expression of M2 and M4 receptors was confirmed in the ileum of M2 and M4 receptor KO mice, respectively (Fig. 5).

We next investigated the localization of the M2 and M4 muscarinic receptors in the mouse ileum by immunohistochemistry using a monoclonal rat IgG antibody for the mouse M2 receptor and a polyclonal rabbit IgG antibody for the M4 receptors. The strong immunoreactivity of an anti-M2 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. 6, A and B). We then stained the mouse ileal tissue with antibodies that 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. 7A). Double staining with an anti-M2 receptor antibody and these neuron markers produced a prominent yellow signal in the myenteric plexus and muscular layers (Fig. 6, A and B), indicating the localization of M2 receptors in neurons. In contrast, an immunoreactivity of M3 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 M4 receptor antibodies was also stained with a 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, whereas that of anti-M4 receptor antibody was observed (Fig. 6D). Immunoreactivity of the anti-M3 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, immunoreactive 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 co-localization of both receptors in myenteric neurons. In the myenteric plexus of M4 receptor KO mice, 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

**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>
<thead>
<tr>
<th></th>
<th>Amount of Released ACh, pmol/g tissue/min</th>
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<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>M2/M4-KO</td>
<td>11</td>
</tr>
<tr>
<td>M2/M3-KO</td>
<td>10</td>
</tr>
</tbody>
</table>

Values are expressed as mean ± SE. EFS, electrical field stimulation; ACh, acetylcholine; LMMP, longitudinal muscle with myenteric plexus; KO, knockout. *Significantly different from the value in the wild-type mice noted in Table 1 at $P < 0.05$ (Student’s t-test).
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 this 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 mice, 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), 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 ACh release in

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**FIG. 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. Tissue sections (A) and whole-mount preparations (B–D) were treated with the anti-M2 receptor antibody and stained with Alexa Flour 568-conjugated anti-rat IgG antibody (red). Co-localization is visualized as yellow in the merged images. A: images of double staining with anti-M2 receptor antibody and anti-neurofilament, -synaptophysin, and -glial fibrillary acidic protein (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.
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 mice compared with wild-type mice. The finding of RT-PCR revealed the expression of M2 and M4 receptors in the ileum of wild-type mouse. Furthermore, 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 studied. In this 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,b; 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 M4 receptors has been shown 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 this 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 show 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

**FIG. 7.** Localization of M4 receptors in the ileum of wild-type and M4 receptor KO mice. Whole-mount preparations were treated with the anti-M4 receptor antibody and stained with FITC-conjugated anti-rabbit IgG antibody. Co-localization is visualized as yellow in the merged images. **A:** images of double staining with anti-M4 receptor and anti-synaptophysin antibodies in the CM and MP layers. 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:** image of anti-M4 receptor antibody in MP of M4 receptor KO mouse. Scale bars, 50 μM.
with the report that M₃ 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 CNS, 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-M₁ antibody. In this study, co-localization 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 this study. In wild-type mice, 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 seems that the loss of either receptor was functionally compensated by a remaining receptor.

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