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Abstract: Functional roles of muscarinic acetylcholine receptors in the regulation of mouse stomach motility were examined using mice genetically lacking muscarinic M2 receptor and/or M3 receptor and their corresponding wild-type (WT) mice. Single application of carbachol (1 nM-10 □ M) produced concentration-dependent contraction in antral and fundus strips from muscarinic M2 receptor knockout (M2R-KO) and M3 receptor knockout (M3R-KO) mice but not in those from M2 and M3 receptors double knockout (M2/M3R-KO) mice. A comparison of the concentration-response curves with those for WT mice showed a significant decrease in the negative logarithm of EC50 (pEC50) value (M2R-KO) or amplitude of maximum contraction (M3R-KO) in the muscarinic receptor-deficient mice. The tonic phase of carbachol-induced contraction was decreased in gastric strips from M3R-KO mice. Antagonistic affinity for 4-diphenylacetoxy-N-methyl-piperidine (4-DAMP) or 11-([2-[(diethylamino)methyl]-1-piperdinyl]acetyl)-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one (AF-DX116) indicated that the contractile responses in M2R-KO and M3R-KO

mice were mediated by muscarinic M3 and M2 receptors, respectively. Electrical field stimulation (EFS, 0.5-32 Hz) elicited frequency-dependent contraction in physostigmine- and N □-nitro-L-arginine methylester (L-NAME)-treated fundic and antral strips from M2R-KO and M3R-KO mice, but the cholinergic contractile components decreased significantly compared with those in WT mice. In gastric strips from M2/M3R-KO mice, cholinergic contractions elicited by EFS were not observed but atropine-resistant contractions were more conspicuous than those in gastric strips from WT mice. Gastric emptying in WT mice and that in M2/M3R-KO mice were comparable, suggesting that motor function of the stomach in the KO mice did not differ from that in the WT mice. The results indicate that both muscarinic M2 and M3 receptors but not other subtypes mediate carbachol- or EFS-induced contraction in the mouse stomach but that the contribution of each receptor to concentration-response relationships is distinguishable. Although there was impairment of nerve-mediated cholinergic responses in the stomach of KO mice, gastric emptying in KO mice was the same as that in WT mice probably due to the compensatory enhancement of the non-cholinergic contraction pathway.

Functional roles of muscarinic M_2 and M_3 receptors in mouse stomach motility: studies with muscarinic receptor knockout mice

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Short title. Muscarinic receptor subtypes in mouse stomach

Abstract

Functional roles of muscarinic acetylcholine receptors in the regulation of mouse stomach motility were examined using mice genetically lacking muscarinic M_2 receptor and/or M_3 receptor and their corresponding wild-type (WT) mice. Single application of carbachol (1 nM-10 μM) produced concentration-dependent contraction in antral and fundus strips from muscarinic M_2 receptor knockout (M_2R-KO) and M_3 receptor knockout (M_3R-KO) mice but not in those from M_2 and M_3 receptors double knockout (M_2/M_3R-KO) mice. A comparison of the concentration-response curves with those for WT mice showed a significant decrease in the negative logarithm of EC_{50} (pEC_{50}) value (M_2R-KO) or amplitude of maximum contraction (M_3R-KO) in the muscarinic receptor-deficient mice. The tonic phase of carbachol-induced contraction was decreased in gastric strips from M_3R-KO mice. Antagonistic affinity for 4-diphenylacetoxy-N-methyl-piperidine (4-DAMP) or 11-([2-[(diethylamino)methyl]-1-piperdinyl]acetyl)-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one (AF-DX116) indicated that the contractile responses in M_2R-KO and M_3R-KO mice were mediated by muscarinic M_3 and M_2 receptors, respectively. Electrical field stimulation (EFS, 0.5-32 Hz) elicited frequency-dependent contraction in physostigmine- and N^{ω}-nitro-L-arginine methylester (L-NAME)-treated fundic and antral strips from M_2R-KO and M_3R-KO mice, but the cholinergic contractile components decreased significantly compared with those in WT mice. In gastric strips from M_2/M_3R-KO mice, cholinergic contractions elicited by EFS were not observed but atropine-resistant contractions were more conspicuous than those in gastric strips from WT

mice. Gastric emptying in WT mice and that in M_2/M_3R-KO mice were comparable, suggesting that motor function of the stomach in the KO mice did not differ from that in the WT mice. The results indicate that both muscarinic M_2 and M_3 receptors but not other subtypes mediate carbachol- or EFS-induced contraction in the mouse stomach but that the contribution of each receptor to concentration-response relationships is distinguishable. Although there was impairment of nerve-mediated cholinergic responses in the stomach of KO mice, gastric emptying in KO mice was the same as that in WT mice probably due to the compensatory enhancement of the non-cholinergic contraction pathway.

Key words, muscarinic M2 receptor, muscarinic M3 receptor, knockout mouse, stomach, motility

1. Introduction

Gastric motility of mammals is regulated by complex and mutual interactions among neurotransmitters, hormones and spontaneous muscle contractility. Functional studies using extrinsic nerve and/or intrinsic nerve stimulation have provided evidences of an important role of parasympathetic cholinergic neurons and postsynaptic muscarinic acetylcholine receptors in contraction of gastric smooth muscle (Lundgren, 1983; Mayer, 1987). Muscarinic acetylcholine receptors belong to a family of G protein-coupled receptors that share conserved amino acid sequences and have a predicted structure of seven transmembrane domains. Molecular cloning studies have demonstrated the existence of five mammalian muscarinic receptor subtypes (M_1-M_5; Caulfield and Birdsall, 1998). Analysis of cloned muscarinic receptors expressed in a variety of cell lines has shown that the individual muscarinic receptor subtypes preferentially interact with specific signal transduction pathways. Muscarinic M_1, M_3 and M_5 receptors couple with $G_{q/11}$ proteins and stimulate phosphoinositide turnover, resulting in production of inositol-trisphosphate (IP_3) and diacylglycerol, while muscarinic M_2 and M_4 receptors couple with $G_{i/o}$ proteins and inhibit adenylate cyclase activity to decrease cytoplasmic cyclic AMP levels (Caulfield and Birdsall, 1998).

Of the five muscarinic acetylcholine receptor subtypes, both M_2 and M_3 types are abundantly expressed in smooth muscles throughout the gastrointestinal tract (Eglen et al., 1996; Ehlert et al., 1997). Radioligand binding (Giraldo et al., 1988), mRNA hybridization (Maeda et al., 1988) and immunoprecipitation studies (Wall et al., 1992) have shown that

muscarinic M_2 receptor is the more abundant of the two receptors (M_2:M_3= 3:1 to 5:1). However, in spite of the dominant expression of the M_2 type, pharmacological analysis of carbachol-induced contraction using muscarinic receptor antagonists indicates involvement of the muscarinic M_3 receptor in contractile responses (Ehlert et al., 1997). The contribution of the muscarinic M_2 receptor to contraction in normal gastrointestinal smooth muscle has only been indirectly demonstrated in experiments which muscarinic M_3 receptors were inactivated by 4-DAMP mustard in the presence of an muscarinic M_2 receptor-selective antagonist and muscle preparations were pretreated with a contractile agent (histamine) and subsequently with cyclic AMP-elevating agents (isoproterenol or forskolin). In these experimental conditions, muscarinic M_2 receptor may be involved in inhibition of isoproterenol- or forskolin-induced relaxation by decreasing the adenylate cyclase activity (Thomas et al., 1993; Sawyer and Ehlert, 1998). Additionally, since the G proteins coupled with muscarinic M_2 receptors ($G_{i/o}$) are sensitive to pertussis toxin, inhibition of muscarinic agonists-induced gastric smooth muscle contraction by pertussis toxin suggests the involvement of muscarinic M_2 receptors in contractile responses (Wrzos et al., 2004).

Heterogeneous expression of muscarinic receptor subtypes in gastrointestinal smooth muscles and lack of subtype-selective muscarinic receptor agonists and antagonists hinder analysis of the functional roles of muscarinic M_2 and M_3 receptors in the regulation of gastric motility. Recently, mutant mice lacking the muscarinic M_2 (M_2R-KO) or M_3 receptor (M_3R-KO) or both the M_2 and M_3 receptors (M_2/M_3R-KO) have been generated by the use of gene targeting techniques. Analysis of these mutant mice has provided clear evidence for the important roles of specific muscarinic receptors in salivary glands, atria,

gastrointestinal tract, urinary bladder, gallbladder and airway smooth muscles (Yamada et al., 2001; Matsui et al., 2000, 2002; Stengel et al., 2000; 2002; Stengel and Cohen, 2002; Struckmann et al., 2003). Although carbachol-induced contraction of the gastric fundus, urinary bladder and tracheal smooth muscles was decreased in the M_3R-KO mice, 50-60% of contractile response still remained, probably due to activation of the M_2 receptor (Stengel et al., 2002). In M_2R-KO mice, carbachol was significantly less potent in producing contraction of the gastric fundus (Stengel et al., 2000). These findings indicate the contribution of both muscarinic M_2 and M_3 receptor activation to exogenously applied carbachol. Although there is no abnormality of motility (diarrhea or constipation) in M_3R-KO mice (Matsui et al., 2000; Yamada et al., 2001), the decrease in contractile responses to carbachol suggests dysfunction of parasympathetic nerve-mediated regulation of gastric motility in KO mice. However, there have been no functional studies on the effects of endogenous acetylcholine on gastric contractility and changes in gastric emptying in muscarinic receptor KO mice.

Therefore, in the present study, we compared the effects of carbachol and EFS on the contractility of stomach strips (antrum and fundus) isolated from M_2R-KO, M_3R-KO and M_2/M_3R-KO mice with their corresponding WT strains. In addition, gastric emptying in WT mice and that in M_2/M_3R-KO mice were compared to determine possible changes in gastric function.

2. Materials and methods

2.1 Animals and tissue preparations

All experiments described were performed in accordance with institutional guidelines approved by the Animal Ethics Committee of the School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido, Japan.

The generation of mice generally lacking muscarinic M_2 or M_3 receptors or both M_2 and M_3 receptors has been described previously (Gomeza et al., 1999; Yamada et al., 2001; Struckmann et al., 2003). The genetic background of the mice used in the present study was 129J1 (50%) x CF1 (50%) for the M_2R-KO and their corresponding WT mice, 129vEv (50%) x CF1 (50%) for the M_3R-KO and their corresponding WT mice, 129J1(25%) x 129SvEv (25%) x CF1 (50%) for the M_2/M_3R-KO mice. The animals were housed in polycarbonate-ventilated cages. The temperature of the animal room was maintained at 23±1℃ with relative humidity of 40-60% and a daily light/dark cycle (7:00am-7:00pm). Food (CRF-1, Oriental Yeast Co Ltd, Japan) and water were given *ad libitum.*

Mice of either sex, aged more than 3 months and weighing 23-30 g, were killed by cervical location. The whole stomach of each mouse was then quickly excised and placed in an ice-cold Krebs solution. Circular muscle strips, freed from mucosa (10-15 mm in length and 0.5 mm in width) were cut from both the fundic and antrum regions. Muscle preparations were suspended vertically in an organ bath filled with Krebs solution (NaCl, 118 mM; KCl, 4.75 mM; $MgSO_4$, 1.2 mM; KH_2PO_4, 1.2 mM; $CaCl_2$, 2.5 mM; $NaHCO_2$, 25 mM and glucose, 11.5 mM) warmed at 37℃ and gassed with 95%O_2 + 5%CO_2 .

2.2 Isometric tension recording

After one-hour equilibration at initial tension of 0.5g, muscle strips were stimulated by 80 mM hyperosmotic KCl solution (80 mM K^+) for 5 min at 15-min intervals until reproducible contractions were obtained. Mechanical activity was measured with an isometric force transducer (SB-11T, Nihon Kohden) and recorded on an ink-writing recorder (U-228, Nippon Denshi Kagaku Japan).

To compare the concentration-response relationships of carbachol among gastric smooth muscle strips isolated from WT and KO mice, cumulative and non-cumulative (single) concentration-response curves were established with half log-units ascending concentration increments (1 nM-30 μM). In a single application protocol, each concentration of carbachol was applied for 5 min followed by washing with fresh Krebs solution three or four times. Both the initial phase (greater than any other sequent phase, phasic contraction) and tonic phase (5 min after application) of the contractile responses to carbachol were used for construction of the concentration-response curves. The interval between successive carbachol applications was 15 min. In a cumulative application protocol, carbachol was applied at 1-2-min intervals and concentration-response curves were established using the amplitude of the initial phasic contraction. The cumulative concentration-response curve was reproducible at 45-min interval construction. Concentration-response relationships of carbachol were analyzed using the computer software program Prism 4. The amplitude of contractions among the preparations was normalized by a standard contraction of 80 mM K^+ and expressed as percentage. Based on

non-linear regression, pEC_{50}, amplitudes of maximum contraction (E_{max}) and a slope factor for the curve (Hill slope) were calculated, and they were used as parameters for comparison of the responsiveness to carbachol in preparations from WT mice and KO mice.

The effects of muscarinic receptor antagonists on carbachol-induced contraction were examined to characterize muscarinic receptor subtypes. After obtaining the control concentration-response curve by cumulative application, muscle preparations were treated with an antagonist for 20 min and then concentration-response curves were newly constructed. The antagonist dissociation constant (pK_d) was determined for each antagonist according by the following formula: =log (CR-1)-log[Ant], where [Ant] denotes the mol/l concentration of the antagonist, and CR is the ratio of the EC_{50} value of carbachol in the absence of the antagonist divided by that in the presence of the antagonist.

To examine the involvement of muscarinic M_2 and M_3 receptors in gastric motor activity, EFS-induced responses in preparations from WT and KO mice were compared. Through two platinum rod electrodes placed on the left and right sides of the organ bath, sandwiching the preparations, EFS was applied repetitively in rectangular pulses (0.5 ms in duration, 40 V) at various frequencies (0.5, 1, 2, 4, 8, 16 and 32 Hz) for 15 s at 5-min intervals and the elicited responses were normalized according to the contractile response to 80 mM K^+. Stimulation frequency-response relationships were established in the normal condition and in the presence of physostigmine (300 nM), physostigmine + L-NAME (100 μM) and physostigmine + L-NAME + atropine (3 μM). Cholinergic contractile components in each gastric strip were defined as an atropine-sensitive contraction of L-NAME + physostigmine-treated preparations and expressed as a percentage amplitude of 80 mM

K^+-induced contraction. The EFS-induced contraction in the presence of atropine was considered as non-cholinergic responses.

2.3 Gastric emptying

Before the gastric emptying experiments, mice (M_2/M_3R-KO mice and corresponding WT mice) were deprived of food for 18 h (6:00 pm-12:00 am) with free access to water. Fasted mice had free access to pre-weighed pellets for 2 h (12:00 am-2:00 pm) and were again deprived of food. Food intake was measured by weighing uneaten pellets. Mice were killed by cervical dislocation 30 min after the start of the experiments. Immediately after the stomach had been exposed by laparotomy, the stomach was quickly removed and dry gastric contents obtained from a vacuum freeze-drying system were weighed. Gastric emptying was calculated by the following formula:
Gastric emptying (%)= [1-(dry weight of food recovered from the stomach/weight of food intake)] x 100.

2.4. Chemicals

The following chemicals were used in the present experiments: atropine sulfate (Sigma), carbamylcholine chloride (carbachol, Sigma), physostigmine sulfate (Wako), N^{ω}-nitro-L-arginine methylester (L-NAME, Sigma), tetrodotoxin (Wako), 4-diphenylacetoxy-N-methyl-piperidine methiodide (4-DAMP, Tocris), and

11-([2-[(diethylamino)methyl]-1-piperdinyl]acetyl)-5,11-dihydro-6H-pyrido[2,3-b][1,4]benzodiazepine-6-one (AF-DX116, Tocris). Drugs except for AF-DX116 were dissolved in distilled water. AF-DX116 was dissolved in dimethylsulphoxide, and this solution was diluted with distilled water. The maximum concentration of dimethylsulphoxide in the bathing solution (0.2%) did not change the tonus or spontaneous contractility of muscle strips.

2.5. Statistical analysis

The results of experiments are expressed as means± S.E.M of at least three experiments using muscle strips from different mice. The significance of differences between the values of KO and corresponding WT mice (single comparison) was determined at $P< 0.05$ using Student's *t*-test (paired and unpaired).

3. Results

3.1. Contractile responses to carbachol in stomach strips isolated from WT and KO mice

3.1.1. Fundus

In the present experiments, fundic strips isolated from either WT and KO mice did not show spontaneous contractile activity (Fig.1). Single application of carbachol (1 nM – 3

μM) caused contraction of fundic strips from both M_2R-WT and M_2R-KO mice in a concentration-dependent manner (Figs. 1 and 3A). The contractile response to carbachol consisted of phasic and tonic contractions and was not inhibited by application of the Na^+ channel blocker tetrodotoxin (1 μM, data not shown), suggesting direct action on smooth muscle. pEC_{50}, E_{max} and Hill slope values are shown in Table 1. Although the E_{max} and Hill slope were not different, the pEC_{50} value in M_2R-KO mice (6.51±0.02, n=5) was significantly lower than that in M_2R-WT mice (6.93±0.07, n=5), suggesting a parallel rightward shift of the concentration-response curve (Fig. 3A). When concentration-response curves were constructed using amplitude of tonic contraction (5 min after application), similar pEC_{50} and E_{max} values were obtained in both types of mice (Table 1). Therefore, the contractile response to carbachol was sustained for at least 5 min in both M_2R-WT and M_2R-KO mice. The concentration-response curves for carbachol were also constructed using cumulative application. In contrast to the single application protocol, pEC_{50} values decreased in the cumulative application (M_2R-WT: 6.56±0.06 n=5, M_2R-KO: 6.18±0.05 n=5), but both E_{max} and Hill slope remained essentially unaltered (Table 2). A comparison of the cumulatively constructed concentration-response curves in fundic strips of M_2R-WT and M_2R-KO mice showed that there were no changes in E_{max} and Hill slope, but the pEC_{50} value in M_2R-KO mice was significantly smaller than that in M_2R-WT mice due to a rightward shift of the curve, similar to the case of single dose application (Table 2).

Single application of carbachol (1 nM-10 μM) also caused a concentration-dependent contraction in fundic strips from M_3R-KO mice, but the contractile responses in fundic strips from M_3R-KO mice were not sustained and faded gradually, reaching 20-30% of the

initial peak contraction after 5 min (Fig.1). This kind of decay in the contractile response was not observed in any fundic strips from all WT mice and M_2R-KO mice. Tetrodotoxin did not affect the transient contraction (data not shown). Amplitudes of the initial peak contraction and tonic contraction in the M_3R-KO mice were significantly lower than those in the M_3R-WT mice. However, pEC_{50} values were comparable for fundic strips from M_3R-KO mice and M_3R-WT mice (Fig. 3A, Table 1). A similar decrease in the amplitude of carbachol-induced contraction in the fundus of M_3R-KO mice was also observed when the concentration-response curves were constructed by cumulative application (Table 2).

Although fundic strips from M_2/M_3R-WT mice were sensitive to carbachol, carbachol (1 nM-10 μM) did not cause any contractile responses in fundic strips from M_2/M_3R-KO mice (Figs. 1 and 3, Tables 1 and 2). As indicated in Fig. 1, a small relaxation was produced by a high concentration of carbachol (10 μM), and this relaxation was inhibited by tetrodotoxin (1 μM) or L-NAME (100 μM) (data not shown).

3.1.2. Antrum

In contrast to the fundic strips, antral strips from WT and KO mice showed spontaneous contractile activity which was not different between WT and KO, and among mouse strains. Single application of carbachol caused concentration-dependent contraction in all antral strips from WT mice, M_2R-KO and M_3R-KO mice. The contractile response in the antrum consisted of an initial transient peak (phasic) followed by a sustained contraction (tonic, 60-70% of the peak) superimposed on the spontaneous activity (Fig. 2).

As shown in Fig. 2, the tonic contractile response to carbachol was conspicuously decreased in antral strips from M_3R-KO mice similar to the case in fundic strips. Carbachol did not cause any contractile responses in the antral strips from M_2/M_3R-KO mice. A high concentration of carbachol (10 μM) decreased the spontaneous phasic contraction and muscle tonus. Fig. 3B shows the concentration-response curves for carbachol in the antral strips from the three KO strains and their corresponding WT mice. The parameters calculated from the concentration-response curves are shown in Table 1. Although there were no significant differences in E_{max} and Hill slope, the pEC_{50} value in the antral strips from M_2R-KO mice was significantly lower than that in the antral strips from the corresponding WT mice. On the other hand, E_{max} but not pEC_{50} and Hill slope in the M_3R-KO was significantly different from the values in the M_3R-WT mice (Table 1 and Fig. 3).

A comparison of concentration-response curves constructed by cumulative carbachol application (M_2R-WT vs. M_2R-KO, M_3R-WT vs. M_3R-KO) showed similar changes in contractile response parameters for carbachol in the antral strips from WT and KO mice (Table 2).

3.2. Effects of muscarinic receptor antagonists

The effects of two subtype-preferring muscarinic receptor antagonists, AF-DX116 (M_2) and 4-DAMP (M_3), on carbachol-induced contraction in gastric preparations from M_2R-KO and M_3R-KO mice were examined. In fundic strips from M_2R-KO mice,

AF-DX116 (1 μM) or 4-DAMP (10 nM) shifted the concentration response curve to the right without affecting E_{max} and increased carbachol EC_{50} values (AF-DX116: 2.3 fold, 4-DAMP: 10.8 fold). The pK_d values for AF-DX116 and 4-DAMP were 5.95±0.07 (n=3) and 9.03±0.13 (n=5), respectively. Both antagonists also inhibited the carbachol-induced contraction of antral strips, and the pK_d values for AF-DX116 (6.18±0.1, n=4) and 4-DAMP (9.25±0.14, n=5) were comparable with those obtained in the fundic strips.

Similar experiments were carried out using antral and fundic strips from M_3R-KO mice. AF-DX116 (1 μM) or 4-DAMP (10 nM) inhibited the carbachol-induced contraction in the fundus and shifted the concentration-response curve to the right without affecting E_{max}. The increase in EC_{50} values by antagonists was 14.1 fold (AF-DX116) and 3.5 fold (4-DAMP). From these resulting increases in EC_{50} values, pK_d values for AF-DX116 and 4-DAMP were calculated to be 7.37±0.13 (n=5) and 8.4±0.11 (n=4), respectively. In the antral strips from M_3R-KO mice, very similar pK_d values (AF-DX116: 7.59±0.05 n=5, 4-DAMP: 8.26±0.16 n=8) for carbachol-induced contraction were obtained.

3.3. Mechanical responses induced by EFS in WT and KO mice

3.3.1. Fundus

For all WT mice, EFS of fundic strips caused frequency-dependent small relaxation between 0.5 Hz and 32 Hz during the stimulation periods and sometimes caused slow contraction after cessation of stimulation (16-32 Hz) (Fig.4). Physostigmine (300 nM), a

cholinesterase inhibitor, changed the relaxant responses to frequency-dependent contraction. L-NAME (100 μM), a nitric oxide synthase inhibitor, enhanced the EFS-induced contraction at high frequencies (8-32 Hz) (Fig. 4). These contractile responses were markedly decreased by atropine (3 μM), but small slowly-developing contractions remained at high frequencies (8-32 Hz) (Fig.4). Tetrodotoxin (1 μM) completely abolished the EFS-induced mechanical responses under all conditions (data not shown). In the present study, cholinergic contractile components were defined as atropine-sensitive EFS-induced responses in physostigmine plus L-NAME-treated preparations and expressed as percentage of 80 mM K^+-induced contraction. As shown in Table 3, relative amplitudes of EFS-induced cholinergic contraction in WT mice were different among three strains examined (M_2R-WT > M_3R-WT > M_2/M_3R-WT).

EFS (0.5-32 Hz) also induced relaxation of fundic strips from M_2R-KO mice. Physostigmine (300 nM) or additional application of L-NAME (100 μM) changed the relaxation to contraction, but the amplitudes of EFS-induced contraction were lower than those in WT mice (Fig. 5A) and cholinergic contractile components were significantly decreased in the M_2R-KO mouse strips (Table 3). Fundic strips from M_3R-KO mice also responded to EFS and frequency-dependent relaxations were evoked under normal conditions. Physostigmine (300 nM) did not change the relaxation to marked contraction (as opposed to M_2R-KO mice), but addition of L-NAME changed the relaxation to contraction, the amplitude of which was comparable to that in fundic strips from WT mice (Fig. 5B). However, the relative amplitude of cholinergic components in the fundus of M_3R-KO mice was also small (Table 3). In contrast to the decrease in cholinergic responses,

EFS-induced contraction (8-32 Hz) in the atropine-treated preparations from M_3R-KO mice was higher than that in the atropine-treated preparations from M_3R-WT (amplitudes of contraction in WT and M_3R-KO mice: 8.3±3.8% and 17.9±4.8% at 8 Hz (P=0.17, WT vs. KO), 18.8±7.4% and 45.6±6.6% at 16 Hz (P=0.03), and 32.6±11.5 % and 63.7±6.7 % at 32 Hz (P=0.06, n=5) (Fig. 5B). EFS also caused frequency-dependent relaxation of preparations from M_2/M_3R-KO mice (data not shown). The addition of L-NAME, but not that of physostigmine, changed the relaxation to a contractile response, but atropine did not decrease the frequency-dependent contraction, suggesting the absence of cholinergic contractile components in the fundus of M_2/M_3R-KO mice (Fig. 5C, Table 3). In contrast to the abolishment of cholinergic components, the amplitudes of atropine-resistant contractions in the fundus of M_2/M_3R-KO mice (12.7±11% at 8 Hz, P=0.18 vs. WT, 20.2±15.4% at 16 Hz, P=0.12 vs. WT and 30±17.6 % at 32 Hz, P=0.039 vs. WT, n=4) were larger than those in the fundus of WT mice (0.8±0.3% at 8 Hz, 2±0.5% at 16 Hz and 3±0.7% at 32 Hz, n=4) (Fig. 5C).

3.3.2. Antrum

A similar comparison of EFS-induced responses was carried out in antral strips from WT and KO mice. In antral strips from WT mice, EFS caused small relaxation or attenuation of spontaneous contraction during the stimulation, but the response changed to a small contraction at 32 Hz (Fig.6). After cessation of EFS, frequency-dependent off-contractions were observed from 4 Hz to 32 Hz. Physostigmine (300 nM) changed the

relaxation to contraction and potentiated the contractile responses at high frequencies (Fig.6). Addition of L-NAME (100 μM) further potentiated the EFS-induced contractions and masked the off-contractions. Atropine (1 μM) almost completely abolished the on-contractions, but off-contractions remained (Fig. 6). Tetrodotoxin (1 μM) abolished both on- and off-responses induced by EFS in all antrum preparations examined (data not shown). Similar with the case in the fundic strips, amplitudes of EFS-induced cholinergic contraction in WT mice were also different among three strains (M_2R-WT = M_3R-WT > M_2/M_3R-WT).

As shown in Fig. 7A, the EFS-induced on-contractions in physostigmine plus L-NAME-treated antrum preparations from M_2R-KO mice were smaller than those in antrum preparations from M_2R-WT mice. The decrease in EFS-induced contraction resulted in a significant decrease in cholinergic contractile components in the antral preparations from M_2R-KO mice (Table 3). Compared with the antral strips from M_3R-WT and M_3R-KO mice, there was no difference in the contractile responses of physostigmine-treated preparations, but the contractile responses of M_3R-KO antral strips in the presence of physostigmine and L-NAME were smaller than those of M_3R-WT mice strips (Fig. 7B). Due to these differences, cholinergic contraction in antral strips from M_3R-KO mice also decreased significantly (Table 3). EFS-induced contractions in antral strips from M_2/M_3R-KO mice in the presence of physostigmine and L-NAME were resistant to atropine (1 μM, Fig. 7C), and cholinergic contractions induced by EFS were not detected at all (Table 3).

3.4. Gastric emptying

Food intake of M_2/M_3R-WT and M_2/M_3R-KO mice over a period of 2h after fasting was 0.89±0.15 g (n=5) and 0.67±0.09 g (n=5) (P=0.25, WT vs. KO). The percentage of food remaining in the stomach (gastric emptying rate) at 30 min after being deprived of food were 61.4±4.7% in WT (n=5) and 60.8± 5.1% (n=5) in KO mice (P=0.93, WT vs. KO). The gastric emptying rates in the WT and KO mice were essentially identical.

4. Discussion

Peripheral muscarinic receptors are involved in the regulation of many important physiological functions, such as control of heart rate, stimulation of glandular secretion, and visceral smooth muscle contraction by parasympathetic nerves. In this study, we used M_2R-KO, M_3R-KO and M_2/M_3R-KO mice to examine the potential roles of muscarinic M_2 and M_3 receptors in gastric smooth muscle contractions induced by cholinergic stimulation. The results showed a decrease in cholinergic nerve (endogenous acetylcholine)-mediated contractions in gastric preparations from M_2R-KO and M_3R-KO mice in addition to a reduction of the responsiveness to carbachol. Cholinergic nerve-mediated and carbachol-induced contractions almost completely disappeared in the gastric preparations from M_2/M_3R-KO mice, indicating that cholinergic contractions in the mouse stomach are mediated by both muscarinic M_2 and M_3 receptors. However, gastric emptying in M_2/M_3R-KO mice was the same as that in M_2/M_3R-WT mice, probably due to

compensation by non-cholinergic pathways regulating gastric emptying.

First, the mechanical responses to carbachol, a nonselective muscarinic receptor agonist, in gastric strips (fundus and antrum) isolated from M_2R-KO, M_3R-KO, or M_2/M_3R-KO mice were characterized. Carbachol was able to produce tetrodotoxin-insensitive contractions in gastric strips from M_2R-KO and M_3R-KO mice. In gastric strips from M_2R-KO mice, the contractile responses to carbachol were antagonized by AF-DX116 (pK_d: 5.95 and 6.18) or 4-DAMP (pK_d: 9.03 and 9.25). These values are consistent with the documented pK_d values for the muscarinic M_3 receptor (Caulfield and Birdsall, 1998; Wang et al., 2004), indicating that the muscarinic M_3 receptor mediates the responses to carbachol in the stomach of M_2R-KO mice. Although the carbachol-induced contractions in preparations from M_3R-KO mice were antagonized by AF-DX116 or 4-DAMP, the pK_d values for AF-DX 116 (7.37 and 7.59) and 4-DAMP (8.26 and 8.4) were comparable to documented pK_d values for the muscarinic M_2 receptor (Caulfield and Birdsall, 1998; Wang et al., 2004), suggesting involvement of the muscarinic M_2 receptor in carbachol-induced contractions in M_3R-KO mice. Carbachol failed to induce contractile responses in both antral and fundic strips from M_2/M_3R-KO mice and instead caused a small relaxation at a high concentration (10 μM). Similar results have been obtained with other gastrointestinal smooth muscle tissues (Matsui et al., 2002; Unno et al., 2005). The small relaxation induced by a high concentration of carbachol was decreased by L-NAME or tetrodotoxin, indicating the involvement of nitric oxide released from enteric neurons in M_2/M_3R-KO mice. This relaxation has been observed in M_3R-KO mice and has been shown to be mediated by muscarinic M_1 receptors present on gastric nitrergic nerves, the

responses of which were masked in gastric preparations from WT mice (Stengel et al., 2003).

Previous studies using gastric fundi from M_2R-KO mice have revealed a rightward parallel shift of the concentration-response curve (decrease in pEC_{50}) (Stengel et al., 2000). On the other hand, in gastric fundi isolated from M_3R-KO mice, there was a marked reduction (50-60%) in maximum contraction (Stengel et al., 2002). Consistent with these observations, the concentration-response curves for carbachol in the fundic strips from M_2R-KO mice shifted 2.6-fold to the right without affecting maximal contraction, and those in fundic strips from M_3R-KO mice shifted downward without changing pEC_{50} values. Although pEC_{50} values and maximal amplitudes of contraction produced by carbachol in the antrum were different from those in fundus, the direction of changes in the contractile parameters for carbachol in the antral strips from M_2R-KO, M_3R-KO and M_2/M_3R-KO mice was almost the same as that obtained in the fundic strips. These results suggest that there were no gastric region-related differences in the contribution of muscarinic M_2 or M_3 receptors to the contractile responses to carbachol. The different changes in the contractile parameters suggest the distinguishable roles of muscarinic M_2 and M_3 receptors in the concentration-response relationships. Deficiency of muscarinic M_2 receptors decreases agonist potency, and deficiency of muscarinic M_3 receptors decreases intrinsic activity of muscarinic agonist, but it is not clear from the present experiments whether contractile responses to carbachol in WT mice (both M_2 and M_3) were a simple addition of muscarinic M_2 and M_3 receptor-mediated responses or whether the two receptors acted synergistically. Muscarinic M_2 receptor activation of pertussis toxin-sensitive G proteins primarily inhibits

adenylate cyclase activity (Griffin and Ehlert, 1992) and opens cationic channels, which causes depolarization and an increase in intracellular Ca^{2+} (Zholos and Bolton, 1997). On the other hand, muscarinic M_3 receptor coupling to the $G_{q/11}$/phospholipase C signaling pathway leads to the generation of IP_3 and diacylglycerol, and these agents increase intracellular Ca^{2+} for M_3-mediated contractions (Caulfield and Birdsall, 1998). The precise relationships between muscarinic M_2 receptor-mediated signal transduction and changes in agonist potency and between muscarinic M_3 receptor-mediated signal transduction and changes in amplitude of contraction remain to be elucidated.

The time course of the carbachol-induced contraction in gastric strips from M_3R-KO mice was clearly different from that in the gastric strips from M_2R-KO mice and all WT mice. Carbachol produced only phasic contractions in gastric strips from M_3R-KO mice but caused phasic and comparable tonic contractions in gastric strips from M_2R-KO and WT mice. The differences suggest the involvement of muscarinic M_3 receptor and coupled signaling mechanisms in the tonic contraction phase. MacCarron et al. (2002) analyzed the phasic and tonic components of agonist-induced smooth muscle contraction and demonstrated that the first component is transient, reflecting Ca^{2+} release from internal stores by IP_3; depletion of the store leads to Ca^{2+} entry via voltage-dependent Ca^{2+} channels, which generates a tonic contractile phase. Therefore each contractile component requires IP_3. An obligatory role of IP_3 in the production of the tonic contractile component was also indicated by the fact that the tonic contraction was inhibited in smooth muscle cells that lack IP_3 receptors (Suzuki et al., 2000). Taken together, the results indicate that IP_3 generated by activation of the muscarinic M_3 receptor is needed to produce both the phasic

and tonic contractions of receptor agonists. In M_3R-KO mice, the lack of muscarinic M_3 receptors resulted in no production of IP_3 and decreased linked phasic and tonic contractions induced by carbachol.

The pharmacological properties of EFS-induced mechanical responses in the fundus suggest the presence of at least three kinds of enteric nerves. One group are the nitrergic nerves, which mediated relaxation under normal conditions, the second one are the cholinergic nerves, which mediated the atropine-sensitive contraction in physostigmine and L-NAME-treated strips, and the last group are the non-cholinergic nerves, which were involved in the slow contraction in atropine-treated preparations. Similar enteric nerves were also demonstrated in the antrum, although non-cholinergic nerves mediated the off-contraction induced by EFS. Amplitude of EFS-induced cholinergic contraction in three strains of WT mice were different both in fundus (M_2R-WT > M_3R-WT > M_2/M_3R-WT) and antrum (M_2R-WT = M_3R-WT > M_2/M_3R-WT), suggesting mouse strain-related different magnitude of neural cholinergic response in stomach. In fundic strips from M_2R-KO and M_3R-KO mice, cholinergic contractile components induced by EFS were markedly decreased compared with those in fundic strips from the corresponding WT mice, suggesting the involvement of both muscarinic M_2 and M_3 receptors in EFS-induced contraction of the fundus. However, atropine-sensitive contractions were not detected in the EFS-induced responses in fundic strips from M_2/M_3R-KO mice. Similar to fundic strips, cholinergic components of on-contraction were also decreased in antral strips from both M_2R-KO and M_3R-KO mice and were almost completely abolished in antral strips from M_2/M_3R-KO mice. Abolishment of the EFS-induced cholinergic components in fundic and

antral strips from M_2/M_3R-KO mice was supported by the ineffectiveness of gastric strips to bath-applied carbachol to produce contraction. These results clearly demonstrated the impairment of cholinergic nerve-mediated responses in the muscarinic receptor KO mice and also suggest important roles of muscarinic M_2 and M_3 receptors in the contractile responses by nerve-released acetylcholine.

Since intraperitoneal injection of atropine delays gastric emptying in mice (Yeung et al., 2001; Kitazawa et al., 2005), acetylcholine released from cholinergic nerves and muscarinic receptors play an important role in regulation in gastric emptying. However, despite the abolishment of cholinergic nerve-mediated contractions of isolated gastric preparations, the gastric emptying rate in M_2/M_3R-KO and the corresponding WT mice was essentially identical. It has been reported that M_3R-KO mice did not display any apparent gastrointestinal complications such as diarrhea, constipation or hemorrhage and that histological examinations did not reveal any abnormality (Matsui et al., 2000). These findings suggest that gastrointestinal functions are regulated by a large number of different neurotransmitters and neuromodulators. In the present study, non-cholinergic contractile components increased in the fundic strips from M_2/M_3R-KO mice, probably due to the increase in the release of non-cholinergic neurotransmitters and/or in the responsiveness of transmitters to smooth muscle. The observed enhancement of non-cholinergic pathways might compensate for the decrease in cholinergic contractile input to the stomach and maintain normal gastric function.

In conclusion, the results of this study using muscarinic receptor KO mice suggest the obligatory roles of both muscarinic M_2 and M_3 receptors in the contraction produced by

bath-applied carbachol, but the contribution of each receptor subtype to concentration-response relationships is different. Although nerve-mediated cholinergic responses were impaired in the stomach of muscarinic receptor KO mice, gastric emptying was normal in the KO mice, probably due to the compensatory enhancement of non-cholinergic pathways.

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Figure legends

Fig. 1

Typical contractile responses to carbachol in fundic strips from M_2R-WT, M_2R-KO, M_3R-KO and M_2/M_3R-KO mice. Single application of four increasing concentrations of carbachol (10 nM, 100 nM, 1 μM and 10 μM) caused a concentration-dependent contraction. 80 mM high-K^+ (80K) –induced contraction was used to normalize the responses to carbachol.

Fig. 2

Typical contractile responses to carbachol in antral strips from M_2R-WT, M_2R-KO, M_3R-KO and M_2/M_3R-KO mice. Single application of four increasing concentrations of carbachol (10 nM, 100 nM, 1 μM and 10 μM) caused a concentration-dependent contraction. 80 mM high- K^+ (80K) –induced contraction was used to normalize the responses to carbachol.

Fig. 3

Comparison of contractile response curves for carbachol in fundic and antrum strips from M_2R-KO(□), M_3R-KO(○), M_2/M_3R-KO (△) mice and their corresponding WT mice (M_2R-WT:■, M_3R-WT:● and M_2/M_3R-WT:▲). Concentration-response relationships were determined by single application of carbachol to the fundus (A) and antrum (B). The amplitude of the contractions is expressed as a percentage of that induced by 80 mM K^+.

Values are means±S.E.M. of 5 muscle strips isolated from 5 different mice. Calculated pEC_{50} values, E_{max} and Hill coefficients are shown in Table 1.

Fig. 4

Representative tracings of mechanical responses of fundic strips from M_2R-WT mice elicited by EFS (●, 0.5-32 Hz) under different conditions: control, in the presence of physostigmine (Phys, 300 nM), in the presence of physostigmine+L-NAME (100 μM), and in the presence of physostigmine+L-NAME+atropine (Atr, 1 μM). EFS (0.5 ms in duration, 40 V) was applied for 15 s at 5-min intervals.

Fig. 5

Comparison of EFS-induced responses in fundic strips from M_2R-KO, M_3R-KO and M_2/M_3R-KO mice and their corresponding WT mice. Relationships between stimulation frequency and responses were determined in the presence of physostigmine (Phy, 300 nM, ■,□), physostigmine+L-NAME (100 μM, ●,○) and physostigmine+L-NAME+atropine (Atr, 3 μM, ▲, △). Open symbols indicate the mechanical responses in fundic strips from KO mice (A: M_2R-KO, B: M_3R-KO, C: M_2/M_3R-KO), and filled symbols indicate those in fundic strips from corresponding WT mice. Amplitudes of EFS-induced contractions were normalized using 80 mM high-K^+-induced contraction and expressed as relative contractions. Values are means±S.E.M. of 5 muscle strips isolated from 5 different mice.

Fig. 6

Representative tracings of mechanical responses of antral strips from M_2R-WT mice elicited by EFS (●, 0.5-32 Hz) under different conditions: control, in the presence of physostigmine (Phys, 300 nM), in the presence of physostigmine+L-NAME (100 μM), and in the presence of physostigmine+L-NAME + atropine (Atr, 1 μM). EFS (0.5 ms in duration, 40V) was applied for 15 s at 5-min intervals.

Fig. 7

Comparison of EFS-induced responses in antral strips from M_2R-KO, M_3R-KO and M_2/M_3R-KO mice and their corresponding WT mice. Relationships between stimulation frequency and mechanical responses were determined in the presence of physostigmine (Phys, 300 nM, ■,□), physostigmine+L-NAME (100 μM, ●,○) and physostigmine+L-NAME+atropine (Atr, 3 μM, ▲, △). Open symbols indicate the mechanical responses in antral strips from KO mice (A: M_2R-KO, B: M_3R-KO, C: M_2/M_3R-KO), and filled symbols indicate those in antral strips from corresponding WT mice. Amplitudes of EFS-induced contractions were normalized using 80 mM high-K^+-induced contraction and expressed as relative contractions. Values are means±S.E.M. of 5 muscle strips isolated from 5 different mice.

Table 1

pEC_{50}, E_{max}, and Hill coefficient for single dose carbachol-induced contraction in fundic and antral strips from M_2R-KO, M_3R-KO or M_2/M_3R-KO mice and their corresponding WT controls.

	Phasic			Tonic		
	pEC_{50}	E_{max}	Hill	pEC_{50}	E_{max}	Hil1
Fundus						
M_2R-WT	6.93±0.07	211.7±36	0.97±0.04	6.81±0.06	211±35.1	1.04±0.09
M_2R-KO	6.51±0.02[a]	215.5±16.7	1.1±0.05	6.42±0.02[a]	233±13	1.05±0.03
M_3R-WT	7.03±0.08	170.5±13.2	1.03±0.09	6.94±0.07	171±10.2	1.09±0.1
M_3R-KO	6.97±0.17	113±8.8[a]	0.84±0.25	7.06±0.24	40.3±18.5[a]	0.71±0.23
M_2/M_3R-WT	6.86±0.13	249±18.9	0.98±0.12	6.83±0.07	260±23.1	0.82±0.1
M_2/M_3R-KO	ND	-14.7±2.4[a]	ND	ND	-11.4±3.8[a]	ND
Antrum						
M_2R-WT	6.6±0.11	117±5.8	0.91±0.13	6.9±0.06	68±8	0.9±0.07
M_2R-KO	6.2±0.05[a]	98.4±3.5	0.93±0.06	6.16±0.06[a]	70±3.8	0.96±0.07
M_3R-WT	6.5±0.21	107±7.2	0.77±0.18	6.48±0.16	54±6.1	0.73±0.14

M_3R-KO	6.9±0.26	68±14.3 [a]	0.88±0.12	7.51±0.17	25±6.12 [a]	0.81±0.19
M_2/M_3R-WT	6.9±0.12	74±7.5	1.03±0.18	6.94±0.19	49±4.5	0.79±0.17
M_2/M_3R-KO	ND	-0.7±0.7 [a]	ND	ND	-1.1±0.7 [a]	ND

Each value represents the mean±S.E.M. of 5 experiments. Responses to carbachol were characterized using tension at initial peak (within 1 min, phasic) and 5 min later (tonic). E_{max} values are expressed as % of 80 mM K^+-induced contraction. In M_2/M_3R-KO mice, carbachol failed to produce a contraction and pEC_{50} values and slope factor could not be determined (ND). Hill coefficients indicated were not significantly different from unity. a; significantly different from the values of corresponding WT mice ($P<0.05$).

Table 2

pEC_{50}, E_{max}, and Hill coefficient for cumulatively applied carbachol-induced contraction in fundic and antral strips from M_2R-KO, M_3R-KO or M_2/M_3R-KO mice and their corresponding WT controls.

	pEC_{50}	E_{max}	Hill
Fundus			
M_2R-WT	6.56±0.06	214±15.4	0.96±0.07
M_2R-KO	6.18±0.05[a]	245±13.8	0.89±0.05
M_3R-WT	6.67±0.06	182.7±9.7	1.01±0.07
M_3R-KO	6.73±0.1	90.3±7.8 [a]	1.07±0.15
M_2/M_3R-WT	6.7±0.09	259±30	1.1±0.11

M_2/M_3R-KO	ND	-3.4±1.6 [a]	ND
Antrum			
M_2R-WT	6.82±0.13	87±6.4	1.34±0.3
M_2R-KO	6.08±0.09 [a]	75±4.1	1.23±0.13
M_3R-WT	6.92±0.11	74.1±2.6	1.5±0.32
M_3R-KO	7.1±0.16	42±6.9 [a]	0.84±0.18
M_2/M_3R-WT	7.27±0.11	72±4.6	1.03±0.18
M_2/M_3R-KO	ND	-0.26±0.26 [a]	ND

Each value represents the mean±S.E.M. of 5 experiments. E_{max} values are expressed as % of 80 mM K^+-induced contraction. In M_2/M_3R-KO mice, carbachol failed to produce a contraction and pEC_{50} values and slope factor could not be determined (ND). Hill coefficients indicated were not significantly different from unity. a; significantly different from the values of corresponding WT mice ($P<0.05$).

Table 3

Cholinergic contractile components in EFS-induced mechanical responses of smooth muscle strips isolated from the fundus and antrum of WT and muscarinic receptor KO mice.

	Relative amplitude of cholinergic contraction						
Mice	0.5	1	2	4	8	16	32 Hz
Fundus							
M_2R-WT	19±5	41±12	67±15	94±14	112±10	115±9	112±11

M_2R-KO	11±4	24±8	38±11[a]	56±14[a]	70±13[a]	75±13[a]	80±14
M_3R-WT	18±6	32±7	55±8	73±6	88±5	85±7	84±9
M_3R-KO	8±4	10±5[a]	16±6[a]	33±12[a]	52±16	47±17	45±19
M_2/M_3R-WT	4±1	9±2	19±4	29±3	44±3	55±4	68±10
M_2/M_3R-KO	0±0[a]	0±0[a]	1±1[a]	-1±2[a]	-4±4[a]	-1±1[a]	2±2[a]
Antrum (on-response)							
M_2R-WT	21±5	43±11	64±12	76±9	81±8	87±8	79±9
M_2R-KO	5±1[a]	11±2[a]	19±3[a]	32±5[a]	47±6[a]	60±6[a]	59±7
M_3R-WT	18±6	32±7	56±8	73±6	88±5	85±7	84±9
M_3R-KO	8±4	10±5[a]	16±6[a]	34±12[a]	52±16	47±17	45±19
M_2/M_3R-WT	3±2	6±3	12±4	25±7	33±9	46±10	53±11
M_2/M_3R-KO	3±3	5±3	-1±1[a]	0±2[a]	-3±4[a]	-2±3[a]	-1±2[a]

Each value is the mean±S.E.M. of five individual studies. Amplitude of cholinergic responses were defined as the atropine-sensitive contractile components in physostigmine (300 nM) plus L-NAME (100 μM)-treated gastric muscle preparations isolated from respective WT and KO mice and were expressed as a percentage to 80 mM K^+-induced

contraction. a; significantly different from the values of corresponding WT mice ($P<0.05$).

Fig. 1（Kitazawa et al)

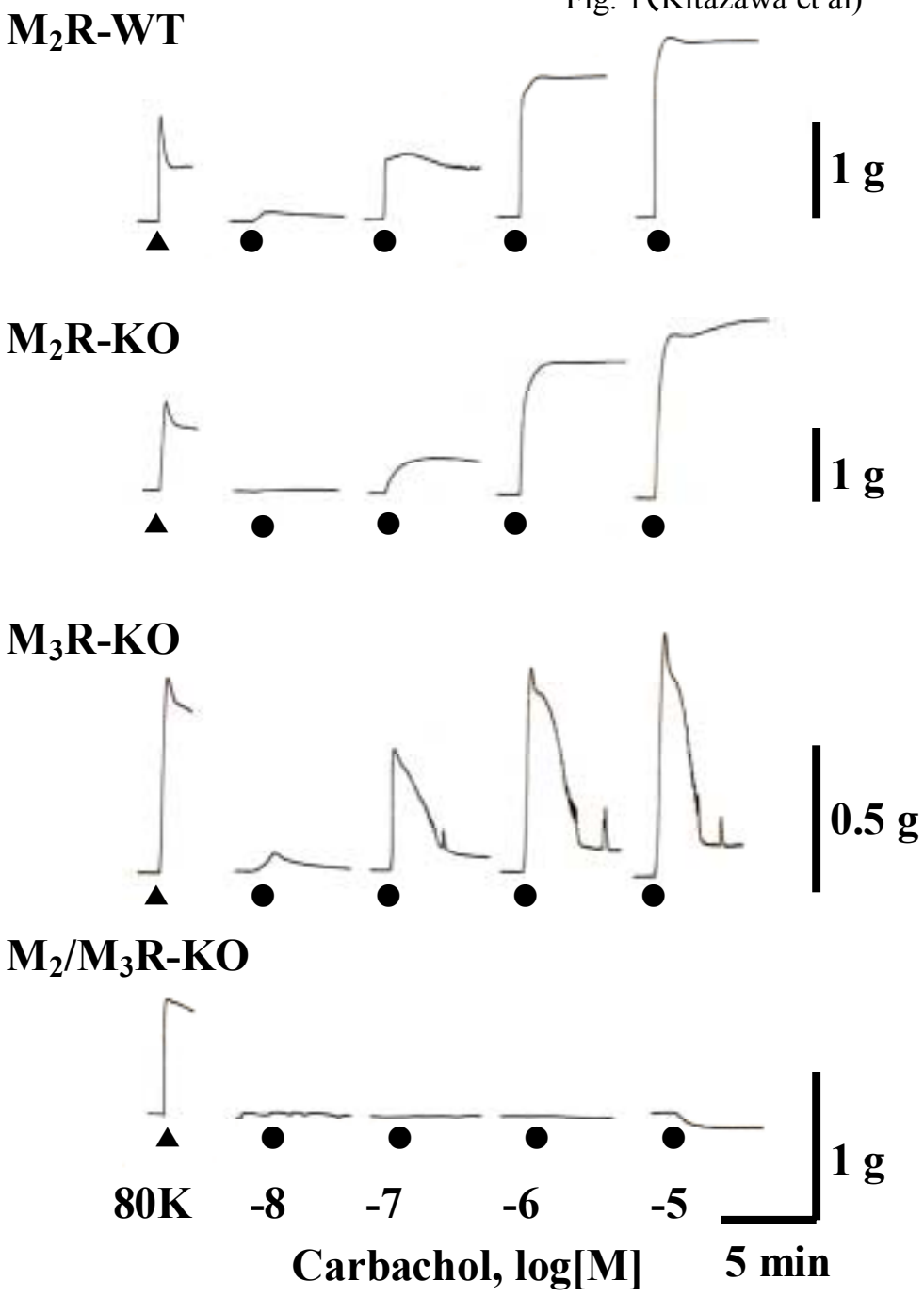

Fig. 2 Kitazawa et al

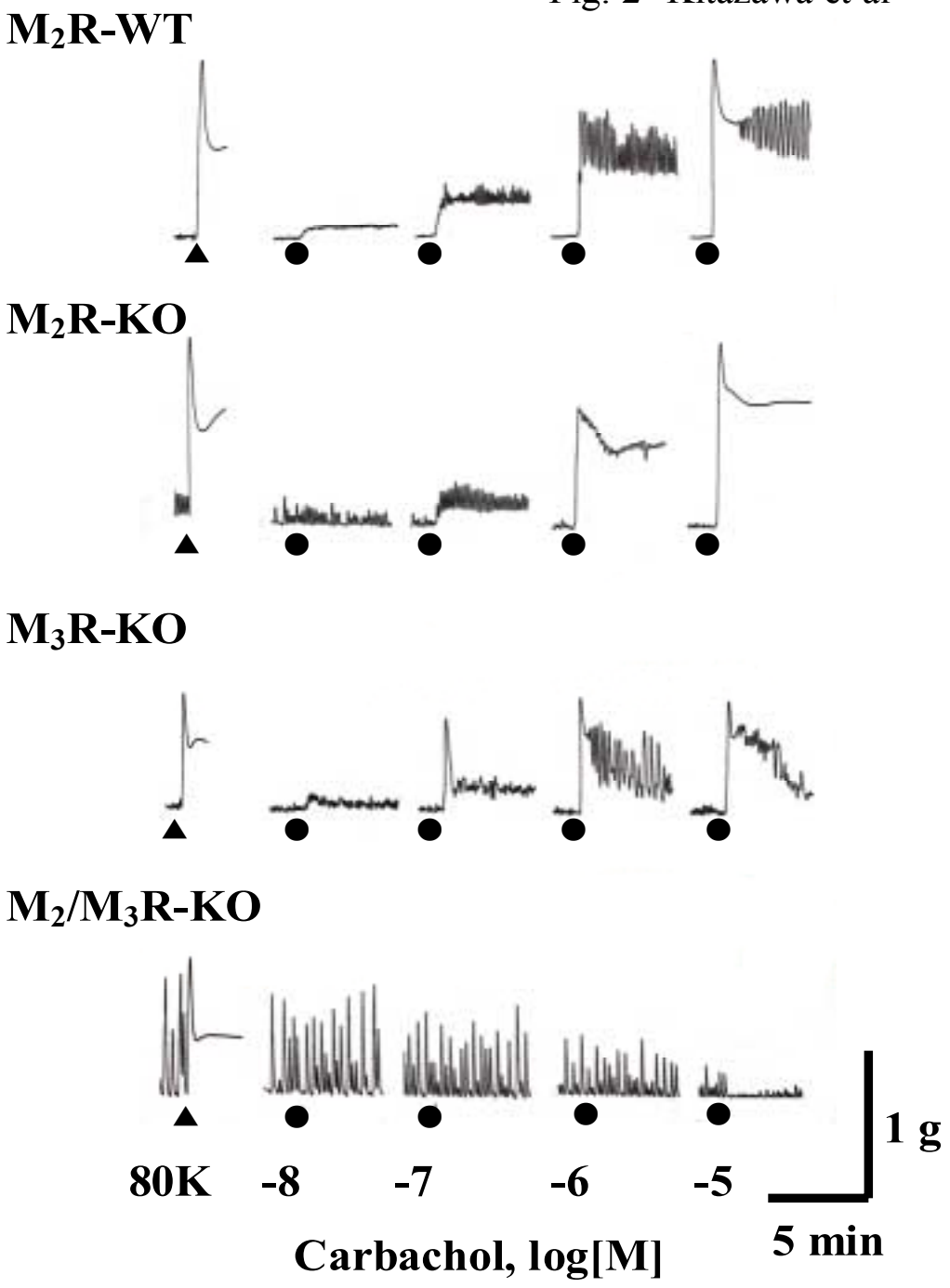

Fig. 3 (Kitazawa et al)

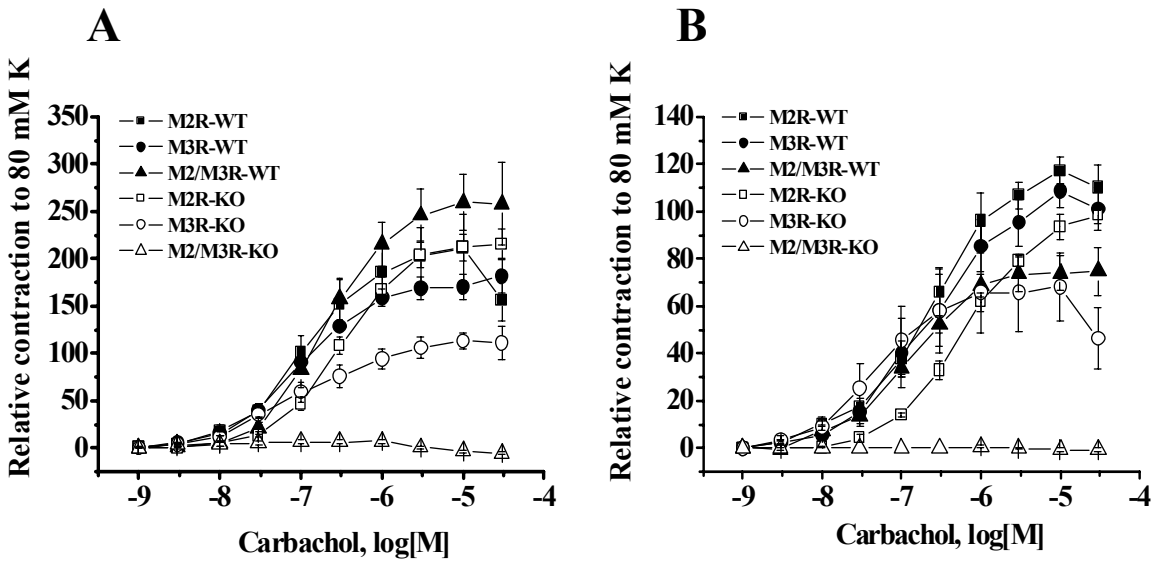

Fig. 4 Kitazawa et al

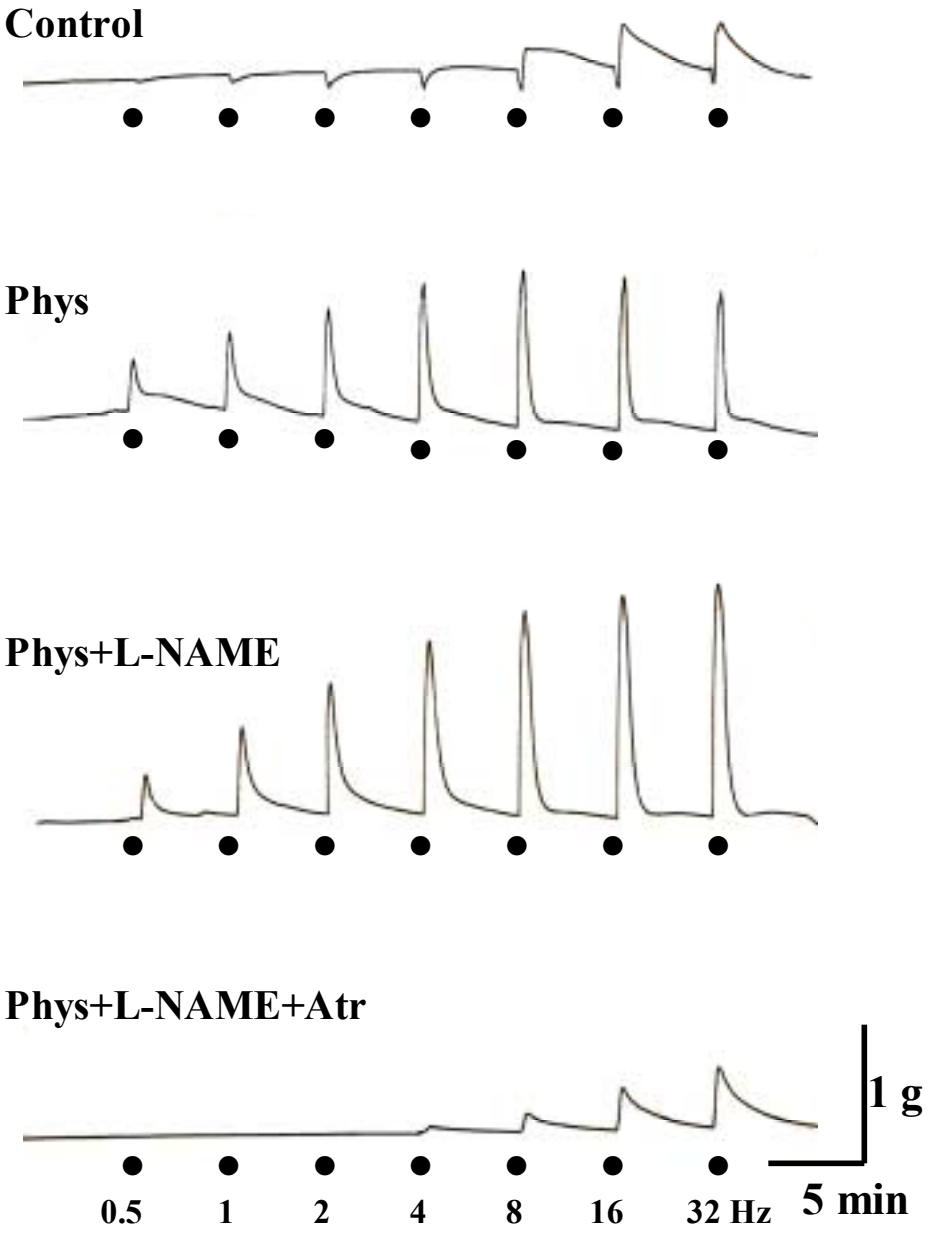

Fig.5 Kitazawa et al

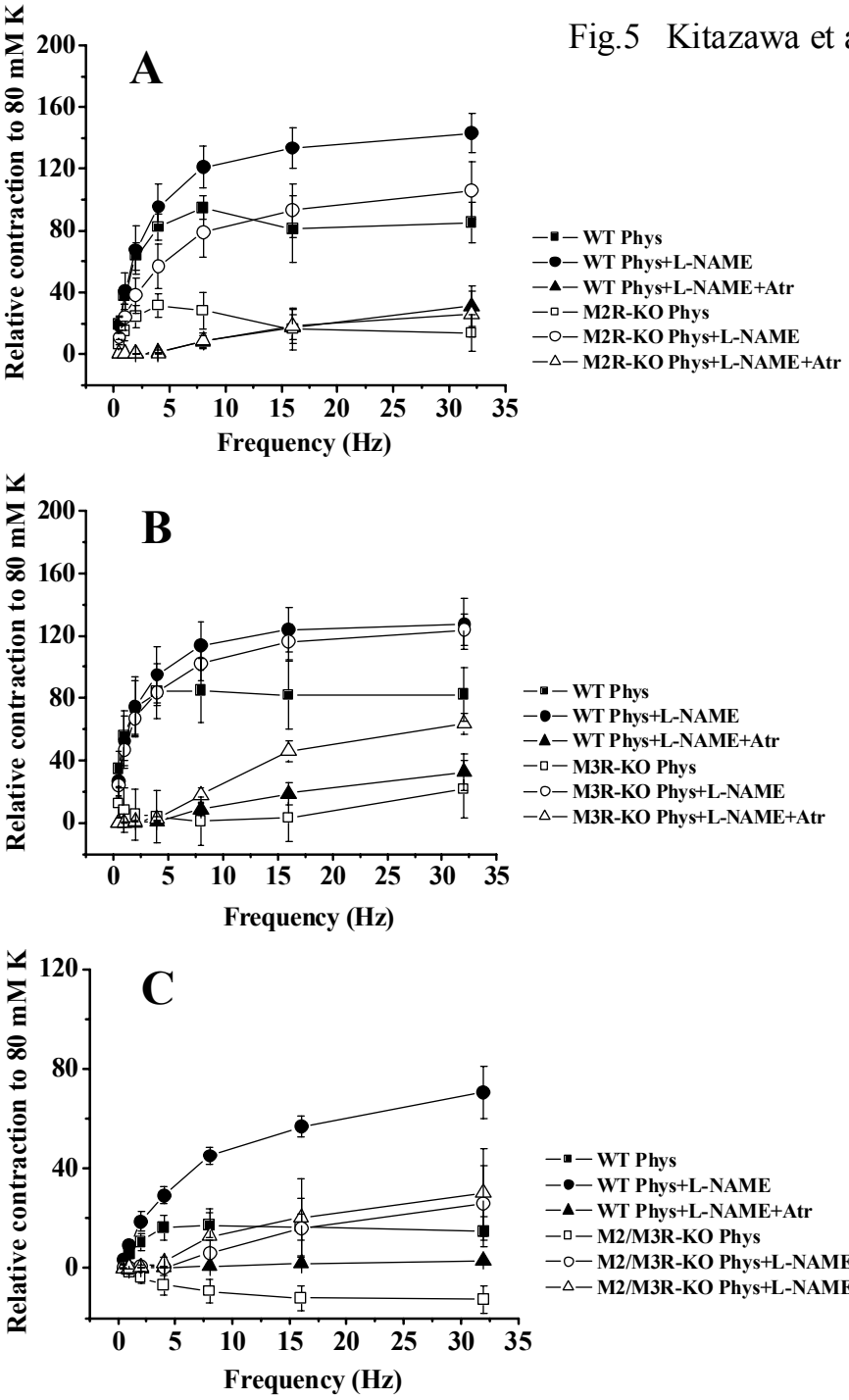

Fig. 6 Kitazawa et al

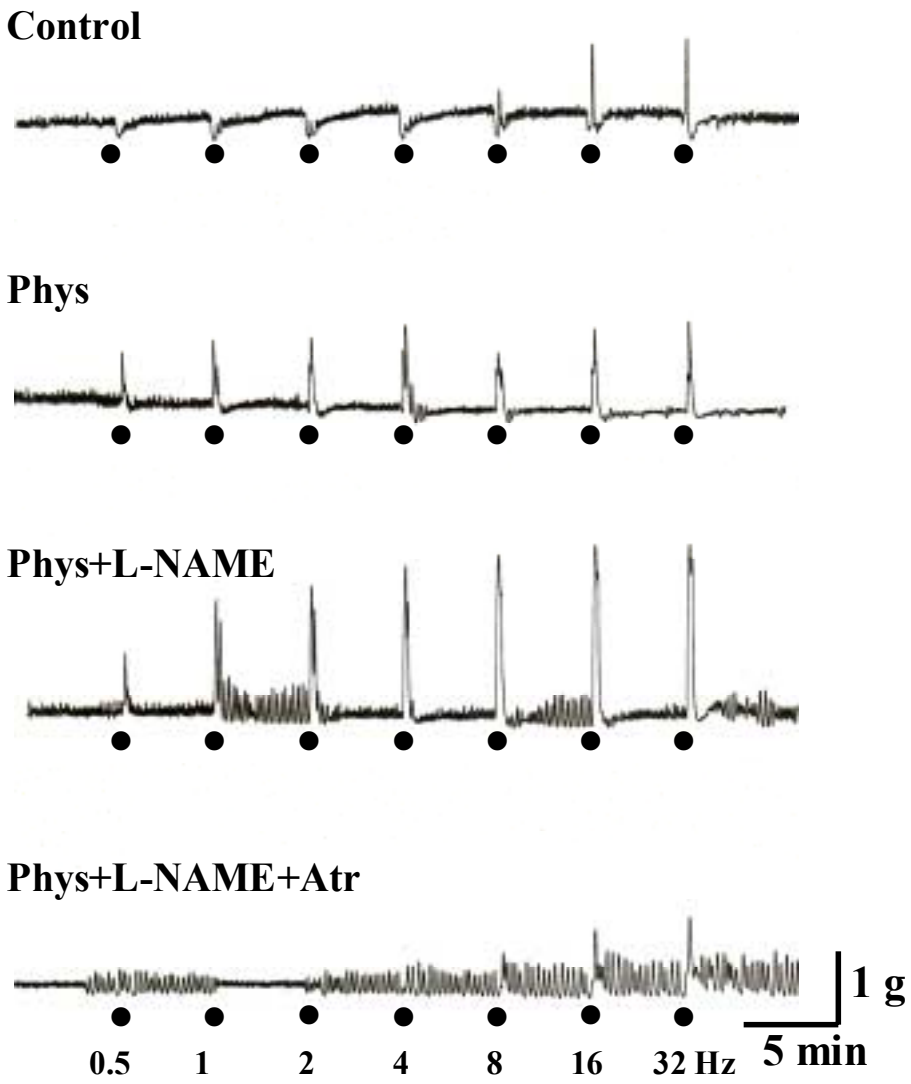

Fig.7 Kitazawa et al