Muscarinic receptor subtypes involved in regulation of colonic motility in mice: functional studies using muscarinic receptor-deficient mice

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Abstract

Although muscarinic M_2 and M_3 receptors are known to be important for regulation of gastric and small intestinal motility, muscarinic receptor subtypes regulating colonic function remain to be investigated. The aim of this study was to characterize muscarinic receptors involved in **regulation of** colonic contractility. M_2 and/or M_3 receptor knockout (KO) and wild-type mice were used in *in vivo* (defecation, colonic propulsion) and *in vitro* (contraction) experiments. Amount of feces was significantly decreased in M_3R-KO and M_2/M_3R-KO mice but not in M_2R-KO mice. Ranking of colonic propulsion was wild-type =M_2R-KO > M_3R-KO > M_2/M_3R-KO. *In vitro*, the amplitude of migrating motor complexes in M_2R-KO, M_3R-KO and M_2/M_3R-KO mice was significantly lower than that in wild-type mice. Carbachol caused concentration-dependent contraction of the proximal colon and distal colon from wild-type mice. In M_2R-KO mice, the concentration-contraction curves shifted to the right and downward. In contrast, carbachol caused **non-sustained** contraction and relaxation in M_3R-KO mice depending on its concentration. Carbachol did not cause contraction but instead caused relaxation of colonic strips from M_2/M_3R-KO mice.

4-[[[(3-chlorophenyl)amino]carbonyl]oxy]-N,N,N-trimethyl-2butyn -1-aminium chloride (McN-A-343) caused a **non-sustained** contraction of colonic strips from wild-type mice, and this contraction was changed to a sustained contraction by tetrodotoxin, pirenzepine

and L-nitroarginine methylester (L-NAME). In the colon of M_2/M_3R-KO mice, McN-A-343 caused only relaxation, which was decreased by tetrodotoxin, pirenzepine and L-NAME. In conclusion, M_1, M_2 and M_3 receptors regulate colonic motility of the mouse. M_2 and M_3 receptors mediate cholinergic contraction, but M_1 receptors on inhibitory nitrergic nerves counteract muscarinic contraction.

Key words: mouse colon, muscarinic receptor, knockout mouse, nitrergic nerves

1. Introduction

Acetylcholine released from parasympathetic nerves plays an important role in regulation of gastrointestinal motility. Muscarinic receptors on enteric neurons and muscle cells are targets for acetylcholine. Molecular cloning studies have demonstrated the presence of five receptor subtypes (M_1-M_5) and co-localization of two or three subtypes in the same organ (Levey, 1993; Eglen et al., 1996; Caulfield and Birdsall, 1998). Although five muscarinic receptors are distributed on enteric neurons and muscle cells of the gastrointestinal tract, M_2 and M_3 are the main receptor subtypes expressed on muscle cells and mediate contraction **induced by** acetylcholine (Eglen et al., 1996; Ehlert et al., 1997; Sawyer and Ehlert, 1998; Eglen, 2001). **The** M_2 receptor is also expressed on enteric cholinergic nerves and regulates acetylcholine release (Vizi et al., 1989; Coulson et al., 2002; Harrington et al., 2010). Immunohistochemical and release studies have indicated that **the** M_1 receptor is localized in myenteric nerves and regulates acetylcholine release (Dietrich and Kilbinger, 1995; Harrington et al., 2007; 2010) and NO release (Wiklund et al., 1993; Iversen et al., 1997). McN-A-343 **has been** used to characterize the M_1 receptor in gastrointestinal tract, but low expression levels of M_1 receptor and **possible actions of** McN-A-343 **on M_2 and M_3 receptors** (Levey, 1993; Richards and Van Giersbergen, 1995; **Ehlert et al., 1999; Figueroa et al., 2009**) hinder analysis of M_1 receptor-mediated actions.

Recently, mutant mice lacking muscarinic receptor subtypes have been generated and these mice have revealed the physiological functions of muscarinic receptors (Wess, 2004). Results of studies using M_2 or M_3 receptor knockout (KO) mice have indicated that M_2 and M_3 receptors cause gastric and intestinal contraction through different mechanisms, but in wild-type mice, a synergistic pathway requiring both subtypes is activated (Unno et al., 2005; Sakamoto et al., 2008). In the stomach of M_3R-KO, M_1-receptor mediated nitrergic relaxation was demonstrated (Stengel and Cohen, 2003). Therefore, muscarinic receptor KO mice are useful for unmasking the functions of muscarinic receptors expressed at low levels. In the colon, migrating motor contractions are regulated by many kinds of enteric neurons, such as excitatory cholinergic, serotonergic and peptidergic neurons, and inhibitory nitrergic neurons (Lyster et al., 1995; Brierley et al., 2001; Powell and Bywater, 2001; Serio et al., 2003; Gourcerol et al., 2009; Dickson et al., 2010). Of cholinergic regulation, stimulation by neostigmine enhances colonic motility in humans through activation of M_1, M_2 and M_3 receptors (Law et al., 2001), and involvement of cholinergic nerves in migrating motor complexes has been **demonstrated** (Brierley et al., 2001; Gourcerol et al., 2009). In the stomach and **ileum,** functional studies have already been carried out with muscarinic receptor KO mice (Unno et al., 2005; Kitazawa et al., 2007), but the function of muscarinic receptor subtypes in the mouse colon remains to be investigated.

In the present study, we used M_2R-KO, M_3R-KO and M_2/M_3R-KO mice and

examined *in vivo* colonic functions (defecation, propulsion) and muscarinic receptor agonist-induced responses of colonic strips. The function of **the** M_1 receptor was further determined **in the KO mice** using McN-A-343.

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 School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido, Japan.

The generation of mice 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 backgrounds of the mice used in the present study were 129J1 (50%) x CF1 (50%) for M_2R-KO and their corresponding wild-type mice, 129vEv (50%) x CF1 (50%) for M_3R-KO and their corresponding wild-type mice, and 129J1(25%) x 129SvEv (25%) x CF1 (50%) for M_2/M_3R-KO mice. DDY mice (25-30 g, males) from Sankyo Lab Service Ltd. (Sapporo, Japan) were also used as control wild-type 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 (7:00 am-7:00 pm). 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 dislocation. **The whole colon was then quickly isolated and placed in an ice-cold Krebs solution. Segments of the proximal colon (20 mm distal to the cecum) and distal colon (20 mm proximal to the anus) were prepared for the experiments**. Muscle preparations **(15-20mm in length)** 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 . Mechanical activity **in the** longitudinal muscle direction was measured with an isometric force transducer (SB-11T, Nihon Kohden) and recorded both on an ink-writing recorder (U-228, Nippon Denshi Kagaku, Tokyo, Japan) and on a computer-aided data acquisition system (Power Lab, Japan Bioresearch Center Nagoya, Japan).

2.2 Isometric tension recording

After 90-min equilibration at an initial tension of 0.5 g, colonic muscle strips were contracted spontaneously and motility patterns were compared between wild-type and muscarinic receptor KO mice. After observing spontaneous contraction patterns, each

muscle strip was stimulated by 50 mM KCl solution (50 mM K^+) for 5 min at 15-min intervals until reproducible contractions were obtained (3-4 times). To compare the concentration-response relationships of carbachol among colonic strips isolated from the wild-type and KO mice, non-cumulative (single) concentration-response curves were established with **half-log unit concentration** increments (1 nM-30 μM). Concentration-response relationships of carbachol were analyzed using the computer software program Origin (Version, 7). The amplitude of contraction (elevation of muscle tonus) among the preparations was normalized by the amplitude of standard contraction of 50 mM K^+ and expressed as percentage. In the present experiments, since there were no differences in the amplitude of 50 mM K^+-induced contraction and responsiveness to carbachol in three wild-type mice and DDY mice, the data from these mice were considered as control responses. The 50 mM K^+-induced contractions in the proximal colon were 0.81±0.08 g (n=15) for wild-type mice, 0.84±0.24 g (n=9) for M_2R-KO mice, 0.68±0.19 g (n=5) for M_3R-KO mice and 0.71±0.08 g (n=7) for M_2/M_3R-KO mice. In the distal colon, the 50 mM K^+-induced contractions were 0.71±0.13 g (n=11) for wild-type mice, 0.71±0.17 g (n=6) for M_2R-KO mice, 0.85±0.19 g (n=5) for M_3R-KO mice and 0.67±0.21 g (n=7) for M_2/M_3R-KO mice, indicating that standard contraction induced by 50 mM K^+ was not different among wild-type and muscarinic receptor KO mice.

Effects of McN-A-343 on isolated colonic contractility were compared between wild-type and M_2/M_3R-KO mice. McN-A-343 caused a **non-sustained** contraction in the

wild-type mouse colon but caused relaxation or reduced spontaneous contractility in the M_2/M_3R-KO mouse colon (see Results). Therefore, McN-A-343-induced **mechanical changes** in the colon **were** evaluated using area **surrounded by contractile curves and baseline (area under the curve, AUC for 5 min)** and normalized by AUC of 50 mM K^+-induced contraction **or by that of the control motility in the absence of drugs.**

2.3. Fecal excretion

Fecal excretion was assessed in mice according to the method described by Izzo et al. (1999). On the day of the experiment, mice were placed individually on a grid floor and given water *ad libitum*. The food was withdrawn at 9:00 am. Three hours later, pellets of feces discharged were collected for 3 h (0.00 pm to 3.00 pm) and they were weighed immediately (wet weight) and then after drying **for 20 h at 50℃ (dry weight)**. An action on secretion or re-absorption of fluids was assessed from the ratio of wet to dry fecal weights (water content, %). Atropine (1 mg/kg) **was** injected intraperitoneally at 0.00 pm and feces were collected for 3 h.

2.4. Colonic propulsion

Colonic propulsion was measured according to the method of Pinto et al. (2002).

After 16-h fasting (9.00 pm – 1:00 pm), a glass bead (2 mm in diameter) was inserted in the colon (20 mm from the anus) of wild-type mice and muscarinic receptor KO mice. The time to expulsion of the glass bead was determined and the times were compared in the animals. In the wild-type mice, the effect of atropine (1 mg/kg, i.p.) on colonic propulsion was examined.

2.5. Chemicals

The following chemicals were used in the present experiments: atropine sulfate (Sigma), carbamylcholine chloride (carbachol, Sigma), methacholine chloride (Sigma), 4-[[[(3-chlorophenyl)amino]carbonyl]oxy]-N,N,N-trimethyl-2butyn-1-aminium chloride (McN-A-343, Sigma), N^{ω}-nitro-L-arginine methylester (L-NAME, Sigma), pirenzepine dihydrochloride (Tocris) and tetrodotoxin (Wako). Drugs were dissolved in distilled water and applied directly to an organ bath.

2.6. Statistical analysis

The results of experiments are generally expressed as means± S.E.M of at least four experiments using muscle strips from different mice. Statistical significance was assessed by Student's t-test or by analysis of variance (ANOVA) followed by

Bonferroni's test using Origin software (Version 7.0, Origin Lab. USA). A *P* value <0.05 was considered to be statistically significant.

3. Results

3.1. Comparison of fecal excretion

Wet weights of feces evacuated over a period of 3 h and mean water contents (%) were 0.17±0.01 g (n=9) and 47% for wild-type mice, 0.14±0.0 2g (n=11) and 46% for M_2R-KO mice, 0.086±0.015 g and 48.6% (n=10) for M_3R-KO mice and 0.06±0.017 g and 46% (n=12) for M_2/M_3R-KO mice, respectively. **Amount of feces decreased** significantly in M_3R-KO and M_2/M_3R-KO mice but not in M_2R-KO mice. **On the other hand,** water contents were the same among all mice examined. In wild-type mice, **wet weight of feces** was reduced by treatment with atropine (1 mg/kg, i.p., 0.03±0.01 g, n=9), **confirming the important role of muscarinic receptors in fecal excretion.**

3.2. Comparison of colonic propulsion

The bead evacuation time was 532±89 s (n=13) in wild-type mice. Atropine (1 mg/kg, i.p.) significantly lengthened the evacuation time (3066±941 s, n=10). Although

the evacuation time was not significantly different in M_2R-KO mice (530±174 s, n=7), the required time to evacuate a bead was significantly longer in both M_3R-KO mice (1730±562 s, n=6) and M_2/M_3R-KO mice (2220±405 s, n=7). Taken together with the defecation results, a significant negative correlation was observed between amount of feces and evacuation time of a bead (***R***=-0.95, ***p***=0.043).

3.3. Spontaneous contraction pattern of colonic strips

Spontaneous contraction observed in proximal and distal colonic strips of wild-type mice could be divided into two patterns according to whether high-amplitude contractions with low frequency were superimposed on high-**frequency** low-amplitude contractions or not. Typical spontaneous motility patterns with high-amplitude contractions (pattern *A*) and without **those** contractions (pattern *B*, only small high-**frequency** contractions) are shown in Fig. 1. In the proximal colon, parameters of high-amplitude (large) contraction were 105±7.1% of 50 mM K^+-induced contraction (amplitude) and 3.11±0.3/10min (frequency), and they were **not significantly different from the parameters in the distal colon**. Small-amplitude and high-frequency basal spontaneous contractions were 38±3% and 5.8±0.3/min (n=22) in the proximal colon and 16±3% and 5.7±0.7/min (n=22) in the distal colon, respectively. Pattern *A* contraction was dominantly observed in the present experimental conditions. In the proximal colon,

percentages of pattern *A* were 73% in wild-type mice (17 of 22 preparations), 71% in M_2R-KO mice (5 of 7 preparations), 80% in M_3R-KO mice (4 of 5 preparations) and 66% in M_2/M_3R-KO mice (6 of 9 preparations). Percentages of pattern *A* in the distal colon were 68% in wild-type mice (15 of 22 preparations), 62% in M_2R-KO mice (5 of 8 preparations), 67% in M_3R-KO mice (4 of 6 preparations) and 33 % in M_2/M_3R-KO mice (2 of 6 preparations). **Amplitude of large** spontaneous contractions in the proximal colon of M_2R-KO, M_3R-KO and M_2/M_3R-KO mice were significantly lower than that in wild-type mice, but the frequencies of contraction were **the same** among **all** colonic preparations. As in the proximal colon, the amplitudes of large spontaneous contraction **in the distal colon of KO mice were** also significantly smaller than that in wild-type **mice** without **change** in frequency (Table 1).

3.4. Carbachol-induced contraction

In the proximal colon of wild-type mice, carbachol caused concentration-dependent contraction (1 nM-100 μM) (Fig. 2). The contractile response to carbachol consisted of phasic and tonic contractions and was not affected by tetrodotoxin (1 μM) (data not shown). The pEC_{50} value and the relative maximum contraction were 6.9±0.12 and 359±41%, respectively (n=7). In M_2R-KO mice, the time course of carbachol-induced contraction was similar to that in wild-type mice (phasic contraction followed by tonic

one). However, the concentration-response curve was shifted both to the right and downward, and the pEC_{50} value (6.34±0.14, n=6) and maximum contraction (238±36%, n=6) were significantly decreased (Fig. 3A). Carbachol also caused contraction in M_3R-KO mice, but the **contraction** was **not sustained** and changed to relaxation at a high concentration (100 μM), resulting in a bell-shaped concentration-response curve. In M_2/M_3R-KO mice, carbachol **did not cause contraction but instead only caused a concentration-dependent relaxation** and decreased spontaneous **rhythmic contraction** (1-100 μM) (Figs. 2 and 3A). The relaxation was abolished by treatment with atropine (1μM) and tetrodotoxin (1 μM) (data not shown).

In distal colon strips, carbachol also caused a concentration-dependent contraction consisting of both phasic and tonic contraction components in wild-type and M_2R-KO mice (Fig. 4). The concentration-response curve for M_2R-KO mice shifted downward (6.03±0.07 and 167±16%, n=6) compared with that for wild-type mice (5.9±0.19 and 257±24%, n=6), and only maximum contraction was decreased significantly. In M_3R-KO mice, carbachol-induced contraction was **not sustained** and relaxation was induced at high concentrations (10-100 μM), resulting **in** a bell-shaped concentration-response relationship. In M_2/M_3R-KO mice, carbachol did not **contract the colonic strips** but instead **only caused a concentration-dependent relaxation** (Figs. 3B and 4).

3.5 Mechanical responses to McN-A-343 in wild-type and M_2/M_3R-KO mice

First, **the effect of McN-A-343 on colonic strips from wild-type mice was examined. As shown in Fig. 5, McN-A-343 caused a non-sustained contraction in both colonic strips, unlike the responses to carbachol (Figs. 2 and 4).** In the presence of tetrodotoxin (1 μM), McN-A-343-induced responses changed from **non-sustained** to sustained contraction, and the contractile responses expressed as AUC increased significantly (Fig. 5 and Table 2). Both in the absence and presence of tetrodotoxin, atropine (1 μM) markedly decreased the McN-A-343-induced contraction (Fig. 5). Pirenzepine (100 nM) was effective for changing the **non-sustained** contraction of McN-A-343 (100 μM) to a sustained contraction, and contractile activity **also** increased significantly (Table 2). **A high** concentration of pirenzepine (10 μM) inhibited the contraction of McN-A-343 (data not shown). Enhancement of McN-A-343-induced contraction by tetrodotoxin and pirenzepine suggests the involvement of an M_1 receptor-linked inhibitory neural pathway activated by McN-A-343. Therefore, the effect of a NO synthase inhibitor, L-NAME, was examined. Similar to the effects of tetrodotoxin and pirenzepine, L-NAME (100 μM) enhanced the McN-A-343-induced responses (Table 2).

In proximal and distal colonic strips from M_2/M_3R-KO mice, McN-A-343 caused concentration-dependent inhibition of muscle contractility. Decreases in resting muscle tension and amplitude of **rhythmic** spontaneous **contraction** were typical responses **to**

McN-A-343 in the proximal colon, but decrease in muscle tension was marked in the distal colon. According to the inhibitory **effects of** McN-A-343, the concentration-responses curves shifted downward compared with those for wild-type mice (Fig. 6). To examine the mechanisms of **the** inhibitory **effects**, the pharmacological properties of McN-A-343-induced responses were assessed. Pirenzepine (100 nM) significantly decreased the MCN-A-343-induced inhibition and reversed it to **contractile responses (Table 3).** Tetrodotoxin and L-NAME also significantly reduced the McN-A-343-induced relaxation. Bethanechol, a muscarinic receptor-selective cholinester, also caused a relaxation of colonic strips, and pharmacological results **similar to those for McN-A-343** were obtained (Table 3).

3.6. Effects of pirenzepine and McN-A-343 on defecation of mice

The outcome of the studies described in the previous paragraph prompted us to examine the effects of pirenzepine and McN-A-343 on defecation of wild-type mice. Pirenzepine (0.04, 0.2 and 1 mg/kg, i.p.), concentration-dependently decreased the amount of feces for 3 h (wet weight, control: 0.3±0.02 g, 0.04 mg/kg: 0.27±0.04 g, 0.2 mg/kg: 0.2±0.04 g, 1 mg/kg: 0.16±0.04 g, n=10). McN-A-343 (1 and 10 mg/kg, i.p.) also decreased the defecation (1 mg/kg: 0.32±0.03 g, 10 mg/kg: 0.2±0.03 g, n=10).

4. Discussion

M_2 and M_3 receptors are the dominant muscarinic receptor subtypes expressed in the gastrointestinal tract (Levey 1993; Eglen et al., 1996; Ehlert et al., 1997; Eglen, 2001). Functional studies using muscarinic receptor-deficient mice have indicated important roles of both M_2 and M_3 receptors in muscarinic agonist-induced contraction of the stomach and ileum (Unno et al., 2005; Kitazawa et al., 2007). Similar to those results, both M_2 and M_3 receptors are involved in the contractile responses to muscarinic agonists in the colon and contribute **to the** propulsive motility of the colon and defecation. In addition, **the** M_1 receptor on enteric nitrergic nerves regulates motility in opposition to M_2/M_3 receptor-mediated colonic contraction.

First, defecation and colonic propulsion were compared *in vivo* using wild-type, M_2R-KO, M_3R-KO and M_2/M_3R-KO mice. Amount of feces tended to decrease in M_2R-KO mice and was significantly decreased in M_3R-KO and M_2/M_3R-KO mice, and atropine decreased feces output in wild-type mice as previously reported (Gourcerol et al., 2009). Comparison of colonic bead evacuation times showed that the ranking order of propulsion force was wild-type = M_2R-KO > M_3R-KO$\geqq$$M_2/M_3$R-KO. Although gastric emptying in M_2/M_3R-KO mice was not different from that in wild-type mice due to compensatory enhancement of a non-cholinergic excitatory pathway (Kitazawa et al., 2007), the present *in vivo* experiments showed a marked decrease in colonic motor

function in M_3R-KO and M_2/M_3R-KO mice. Consequently, the present results indicated a significant role of muscarinic receptors (especially M_3 type) in defecation and colonic propulsion in mice ***in vivo***.

Two patterns of spontaneous contraction in isolated colonic strips were observed in the experiments. One is pattern *A* consisting of **high-frequency** small contractions and superimposed **low-frequency** large contractions (about 3-min intervals), and the other is pattern *B* lacking large contractions. Pattern *A* was dominant in wild-type, M_2R-KO and M_3R-KO mice, but the percentage of appearance for pattern *A* tended to decrease in M_2/M_3R-KO mice (especially **in the** distal colon). Spontaneously occurring migrating motor complexes (Fida et al., 1997; Brierley et al., 2001; Gourcerol et al., 2009) or myoelectric complex (Lyster et al., 1995) have been recorded in the isolated mouse colon and conscious mouse colon. The migrating motor complexes are separated by periods of quiescence and consist of rapid contraction superimposed on a long-duration high-amplitude contraction occurring at 3-min intervals (Fida et al., 1997; Brierley et al., 2001) **similar with the pattern *A* contraction**. **Therefore, the** high-amplitude colonic contractions observed in the present ***in vitro*** study are thought to be consistent with these migrating motor complexes. Both M_2 and M_3 muscarinic receptors are necessary to induce high-amplitude contractions because the amplitude of contraction was significantly decreased in muscarinic receptor KO mice. Brierley et al. (2001) and Gourcerol et al. (2009) have already demonstrated the involvement of cholinergic nerves

and muscarinic receptors in migrating motor complexes. On the other hand, the frequency of high-amplitude contraction was not different in wild-type and muscarinic receptor KO mice, indicating that **M_2 and M_3 receptors are not involved in the regulation of** frequency. A NO synthase inhibitor increased the frequency of giant migrating contraction in the mouse colon (Powell and Bywater, 2001), suggesting that inhibition by nitrergic nerves might suppress the initiation of migrating motor complex **and regulate the frequency**. Atropine decreased both migrating motor complexes and defecation in conscious mice (Gourcerol et al., 2009). Therefore, decrease in the amplitude of large contraction could in part explain the decrease in colonic propulsive ability and following defecation in the muscarinic receptor KO mice in the *in vivo* study. Amplitudes of large contraction were almost the same in muscarinic receptor KO mice, but the colonic propulsive efficacy was not the same between M_2R-KO and M_3R-KO mice. Discrepancy in the results of *in vitro* and *in vivo* studies suggests differences in the regulation of colonic motility by extrinsic parasympathetic nerves from the sacral spinal cord.

Comparison of concentration-response curves for carbachol among wild-type and muscarinic receptor KO mice indicated that M_2 and M_3 receptors, but not other types, are involved in the contraction induced by muscarinic receptor agonists. In M_2R-KO mice, the maximum contraction decreased markedly, but changes in pEC_{50} were different in the proximal colon and distal colon. In M_3R-KO mice, the concentration-response curve

shifted downward and became bell-shaped, similar to that for the stomach of M_3R-KO mice (Stengel and Cohen, 2003), but **a** bell-shaped curve was not the case in the ileum (Unno et al., 2005). The time course of carbachol-induced contraction also changed from sustained (wild-type and M_2R-KO) to **non-sustained (M_3R-KO)** as in gastric preparations (Kitazawa et al., 2007). McCaron et al. (2002) demonstrated that the tonic contractile phase was induced by Ca^{2+} entry from the voltage-dependent Ca^{2+} channel due to inositol-trisphosphate-induced Ca^{2+} store depletion. Therefore, inositol-trisphosphate formation by M_3 receptor activation is necessary for the tonic contraction phase. In M_2/M_3R-KO mice, carbachol did not cause contraction but instead relaxed both colonic strips, which was decreased by atropine and tetrodotoxin. Atropine-sensitive carbachol-induced colonic relaxation in M_2/M_3R-KO mice prompted us to investigate M_1 receptor-mediated actions in the mouse colon. McN-A-343, a **muscarinic receptor agonist, acts on the M_1 receptor with high affinity and high intrinsic activity. However, the affinity and intrinsic activity of McN-343 for M_2 and M_3 receptors are low. In contrast, carbachol expresses almost the same affinity and high intrinsic activity (0.7-1.0) for all muscarinic receptor subtypes (Ehlert et al., 1999; Figueroa et al., 2009). McN-A-343 caused relaxation of the rat small intestine through M_1 receptors (Micheletti et al., 1987; Olgart and Iversen, 1999) but contracted the guinea-pig tenia coli (Hishinuma et al., 1997) and rat colon through M_3 receptors (Borjesson et al., 2000). In the present experiments**, McN-A-343 caused

atropine-sensitive **non-sustained** contractions of the mouse **colon and these contractions were not observed in the M_2/M_3R-KO mice**. Tetrodotoxin changed the **non-sustained** response to McN-A-343 in the wild-type mice colon to **a** sustained one. **Either pirenzepine or L-NAME was also effective in changing the contraction of McN-A-343 into a sustained type as was tetrodotoxin. Since pK_b values of pirenzepine for M_1 and M_3 receptors were reported to be 7.89 and 6.85, respectively (Stengel and Cohen, 2003), 100 nM pirenzepine used was sufficient to block M_1 receptor-mediated action. Taken together these results, the non-sustained colonic contraction induced by McN-A-343 is suggested to be a mixed response composed of smooth muscle contraction (M_2 and M_3 receptors) and relaxation through M_1 receptor mediated inhibitory nitrergic output. According to low affinity and low intrinsic activity of McN-A-343 for M_2 and M_3 receptors (Ehlert et al., 1999; Figueroa et al., 2009), simultaneous activation of M_1 receptor-mediated inhibitory pathway suppresses the M_2/M_3 receptor-mediated sustained contraction and results in a non-sustained contraction shape**. The M_1 receptor-activated nitrergic pathway was demonstrated in M_2/M_3R-KO mice since McN-A-343 caused relaxation of both proximal and distal colon strips, which was inhibited by tetrodotoxin, L-NAME and pirenzepine. Immunohistochemical studies indicated that M_1 receptors are localized on nitrergic neurons in the guinea-pig and human enteric nerves (Harrington et al., 2007; 2010) and that activation of **the** M_1 receptor evoked neural NO release followed by inhibition of

gastrointestinal motility (Iversen et al., 1997; Olgart and Iversen, 1999; Kortezova et al., 2004). Inhibitory effects on intestinal motility by endogenous NO release as a consequence of M_1 receptor activation may represent a muscarinic receptor-mediated negative feedback mechanism of colonic motility. In the human colon, in addition to M_2/M_3 receptor-mediated contraction, activation of **the** M_1 receptor has been shown to be needed to enhance colonic propulsion and movement of luminal contents (Law et al., 2001). Therefore, it is thought that **the** M_1 receptor has an important role in coordinating muscle contraction with other receptor subtypes (M_2/M_3 receptors) in the colon.

The present results suggest an important functional role of **the** M_1 receptor in colonic motor function. However, both McN-A-343 (agonist) and pirenzepine (antagonist) decreased the amount of feces in a dose-dependent manner. Since both drugs affect other muscarinic receptor subtypes depending on the doses (concentrations) and it is difficult to control their concentrations at muscarinic receptors in the colon, we could not evaluate the M_1 receptor-mediated function in this defecation study. Further experiments are needed to clarify the functional relevance of M_1 receptors in the mouse colon ***in vivo***, and M_1 receptor KO mice might be useful for evaluating the regulation of colonic motility by **the** M_1 receptor.

In conclusion, this is the first functional study **on** the role of muscarinic receptor subtypes in colonic motility using M_2/M_3 muscarinic receptor KO mice. Muscarinic M_1, M_2 and M_3 receptors regulate colonic motility of the mouse. M_2 and M_3 receptors mediate

cholinergic contraction, but M_1 receptors on enteric inhibitory nitrergic nerves stimulate NO release counteracting muscarinic contraction.

Acknowledgments

We thank Dr. Jürgen Wess (NIH, Bethesda, MD, USA) for providing the muscarinic receptor mutant mice and for reading the manuscript. This study was supported by a Grant-in-Aid for Scientific Research (No.22380159) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (Komori, S).

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Figure legends

Fig. 1.

Typical spontaneous contractions of proximal and distal colon strips isolated from wild-type mice. Spontaneous contraction observed in colonic strips of the wild-type mouse could be divided into two patterns depending on whether high-amplitude **low-frequency** contractions were superimposed on **high-frequency** low-amplitude contractions (Pattern *A*) or not (Pattern *B*).

Fig. 2.

Typical contractile responses to carbachol in proximal colon strips from wild-type, M_2R-KO, M_3R-KO and M_2/M_3R-KO mice. Single application of five increasing concentrations of carbachol (10 nM, 100 nM, 1 μM, 10 μM and 100 μM, 1-h intervals) caused concentration-dependent contraction in wild-type, M_2R-KO and M_3R-KO mice, but the contraction was **non-sustained** in M_3R-KO mice and the response was changed to relaxation at 100 μM. In M_2/M_3R-KO mice, carbachol caused only relaxation of colonic strips.

Fig. 3.

Comparison of **log concentration-response curves** for carbachol in proximal and distal colonic strips from wild-type (WT, ●), M_2R-KO (○), M_3R-KO (▲) and

M_2/M_3R-KO (△) mice. Concentration-response relationships were determined by single application of carbachol to the proximal colon (A) and distal colon (B). Amplitude of the contraction is expressed as a percentage of that induced by 50 mM K^+. Values are means±S.E.M. of at least 5 muscle strips isolated from 5 different mice. **a: *P*<0.05, b: *P*<0.01, The contractile responses in M_2R-KO mice were significantly different from the corresponding contractions in wild-type mice.**

Fig. 4.

Typical contractile responses to carbachol in distal colon strips from wild-type, M_2R-KO, M_3R-KO and M_2/M_3R-KO mice. Five increasing concentrations of carbachol (10 nM, 100 nM, 1 μM, 10 μM 100 μM) were applied to the organ bath at 1-h intervals, and evoked mechanical responses were observed.

Fig. 5.

Contractile responses to McN-A-343 in isolated muscle strips from the proximal colon and distal colon of wild-type mice. **A:** McN-A-343 (100 μM) caused a **non-sustained** contraction in the proximal colon and distal colon. Tetrodotoxin (TTX, 1 μM) enhanced the McN-A-343-induced contraction in the colon, which was abolished by atropine (1 μM). **B: Effect of TTX (1 μM) on time course of the McN-A-343-induced contraction in the proximal colon (a) and distal colon (b). Amplitude of the contraction is**

expressed as a percentage of that induced by 50 mM K^+. Abscissa is time (sec) after application of McN-A-343 (arrow, 0sec). Values are means±S.E.M. of at least 5 muscle strips isolated from 5 different mice. a: *P*<0.05, b: *P*<0.01, Significantly different from the corresponding control values.

Fig. 6.

Mechanical responses to McN-A-343 in isolated muscle strips from the proximal colon and distal colon of M_2/M_3R-KO mice. A: Typical mechanical responses to McN-A-343 (100 nM-100μM) applied singly at 1-h intervals in the the proximal colon and distal colon from M_2/M_3R-KO mice. B: Comparison of **log concentration-response curves** for McN-A-343 in colonic strips from wild-type mice (●) and M_2/M_3R-KO mice (○). Mechanical responses were evaluated by comparison of AUC (for 5 min) before and after application of McN-A-343. Relative AUC (%) =100 x ((*B*-*A*)/*C*). *A* is AUC before application of **McN-A-343 (control)** and *B* is AUC **after application** of McN-A-343. *C* is AUC of 50 mM K^+-induced contraction. Values are means±S.E.M. of at least 5 muscle strips isolated from 5 different mice. **a: *P*<0.05, b: *P*<0.01, Significantly different from that of wild-type mice.**

Table 1

Comparison of high-amplitude spontaneous contractions observed in the proximal colon and distal colon of wild-type, M_2R-KO, M_3R-KO and M_2/M_3R-KO mice

	Wild-type	M_2R-KO	M_3R-KO	M_2/M_3R-KO
Proximal colon				
Frequency (contractions/10 min)	3.1±0.3 (n=17)	6.6±1.4 (n=5)	3.8±0.7 (n=4)	2.9±0.32 (n=6)
Amplitude (% to 50 mM K^+)	105±7.1 (n=17)	49±6.1[a] (n=5)	58±8.4[a] (n=4)	54±9.9[a] (n=6)
Distal colon				
Frequency (contractions/10 min)	3.4±0.3 (n=15)	5.7±1.6 (n=5)	4.5±0.9 (n=4)	3.8 (n=2)
Amplitude (% to 50 mM K^+)	91.6±8 (n=15)	47±10.6[a] (n=5)	61±15[a] (n=4)	61 (n=2)

Each value is the mean or mean±S.E.M of respective experiments. Amplitude of large spontaneous contraction is indicated as percentage of 50 mM K^+-induced contraction in each colonic strip. **a: *P*<0.05 compared with the wild-type.**

Table 2

Effects of tetrodotoxin, pirenzepine and L-NAME on the contraction induced by McN-A-343 in colonic strips isolated from wild-type mice

	Relative contraction (%)			
	Control	Tetrodotoxin(1 μM)	Pirenzepine (100 nM)	L-NAME(100 μM)
Proximal colon				
10 μM	39.7±7.9	62.5±7.9[a]	66.1±13.8[a]	71.1±16.6[a]
100 μM	42.7±3.7	85.2±13.3[b]	75.4±13.6[b]	64.5±13.3[a]
Distal colon				
10 μM	51.0±8.5	93.0±11.8[a]	106±16[b]	123±31.8[b]
100 μM	63.9±10.4	142.1±26.2[b]	125.4±1.1[a]	141.6±27.9[b]

Values are means ± S.E.M. of over 4 experiments. Contractile responses were normalized by the AUC of 50 mM K^+-induced contraction (for 5 min) and expressed as percentage contraction. **a: *P*<0.05, b: *P*<0.01 compared with the control responses.**

Table 3

Effects of tetrodotoxin, pirenzepine and L-NAME on the relaxation induced by McN-A-343 (10 μM) and bethanechol (100 μM) in colonic strips isolated from M_2/M_3R-KO mice

	Relative relaxation (%)			
	Control	Pirenzepine (100 nM)	Tetrodotoxin (1 μM)	L-NAME(100 μM)
Proximal colon				
McN-A-343	14.7±1.7	-13.7±6.7[b]	4.2±0.83[b]	6.6±1.4[a]
Bethanechol	26.6±3.5	4.4±6.4[a]	5.5±1.6[a]	8.2±3.3[a]
Distal colon				
McN-A-343	21.8±3.8	-6.3±1.9[a]	1.9±0.9[b]	0.8±0.9[b]
Bethanechol	33.2±5.9	4.3±1.2[a]	0.74±1.1[a]	10.9±3.4[a]

Values are means ± S.E.M. of 4 experiments. Relaxation was evaluated by comparison of AUC **of colonic strips** before and after application of muscarinic **receptor** agonists. Relative relaxation = 100 x (1-***B/A***). ***A*** is AUC **(for 5 min)** before application of **agonists (control)** and ***B*** is AUC **after treatment with agonists**. ***B/A*** **indicates change of AUC by agonists. Therefore, a negative value of relative relaxation (*B* > *A*) represents increase in AUC (contraction) by agonists. a: *P*<0.05, b: *P*<0.01 compared with control responses.**

Fig. 1

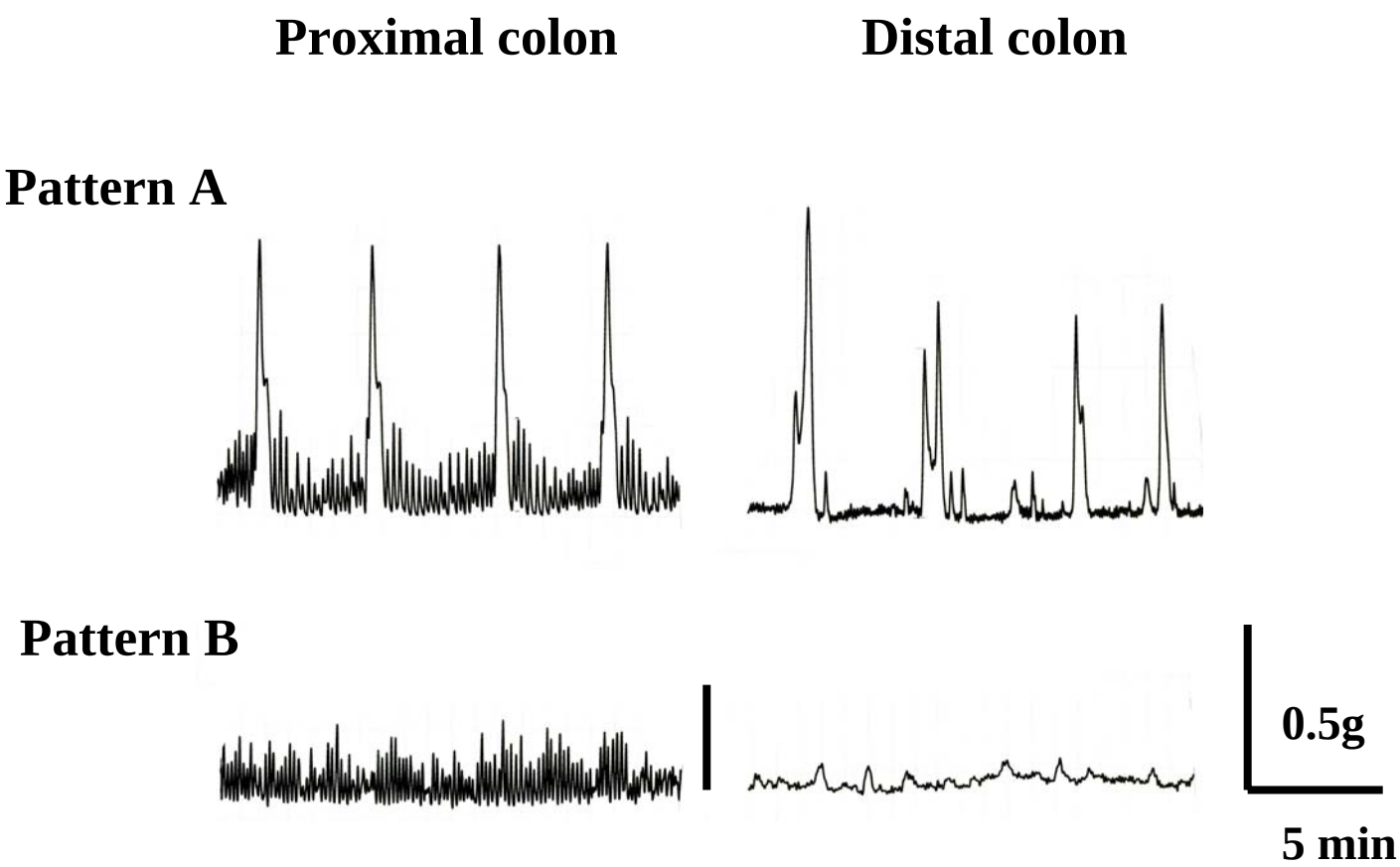


Fig. 2

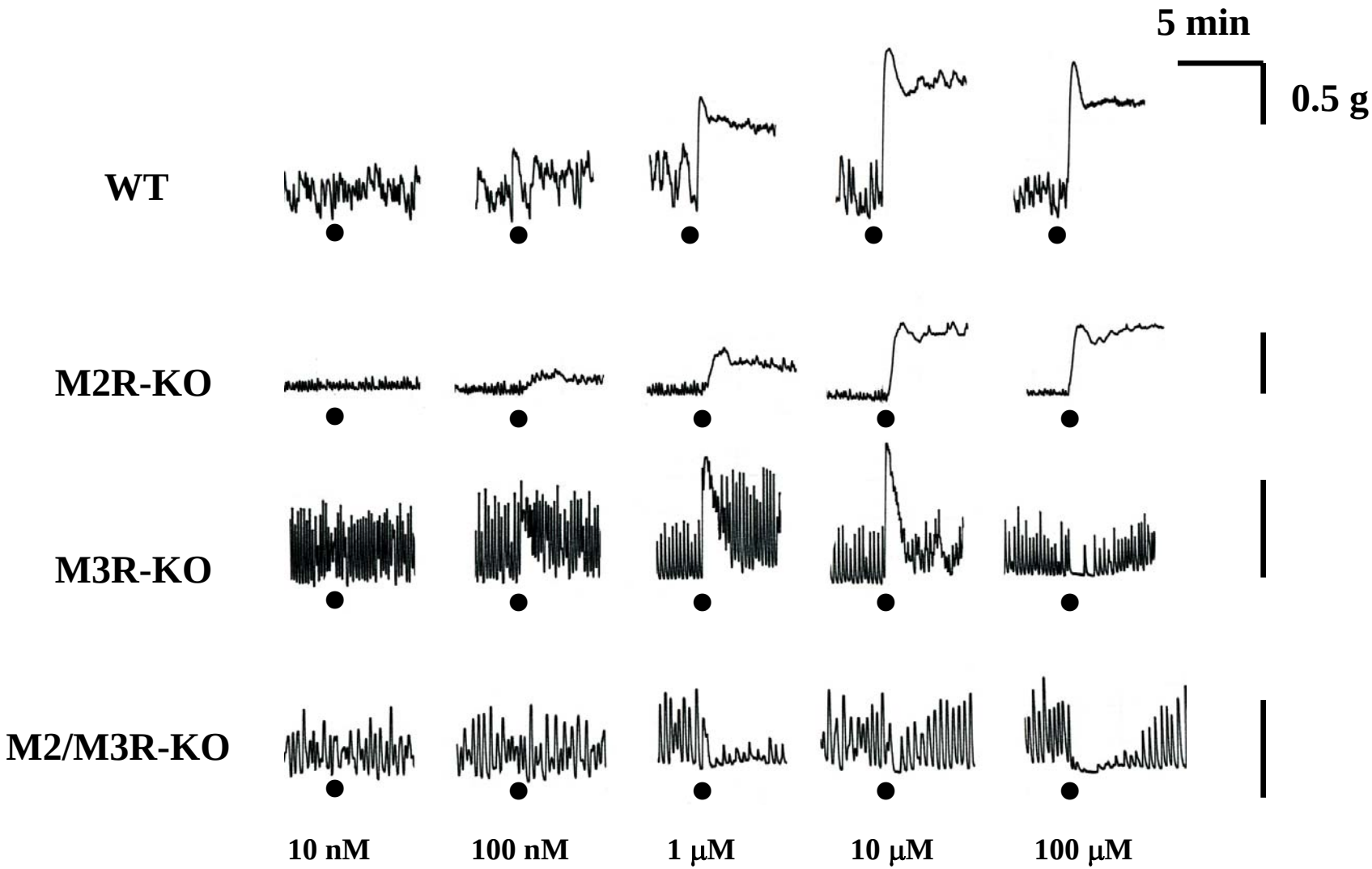


Fig. 3

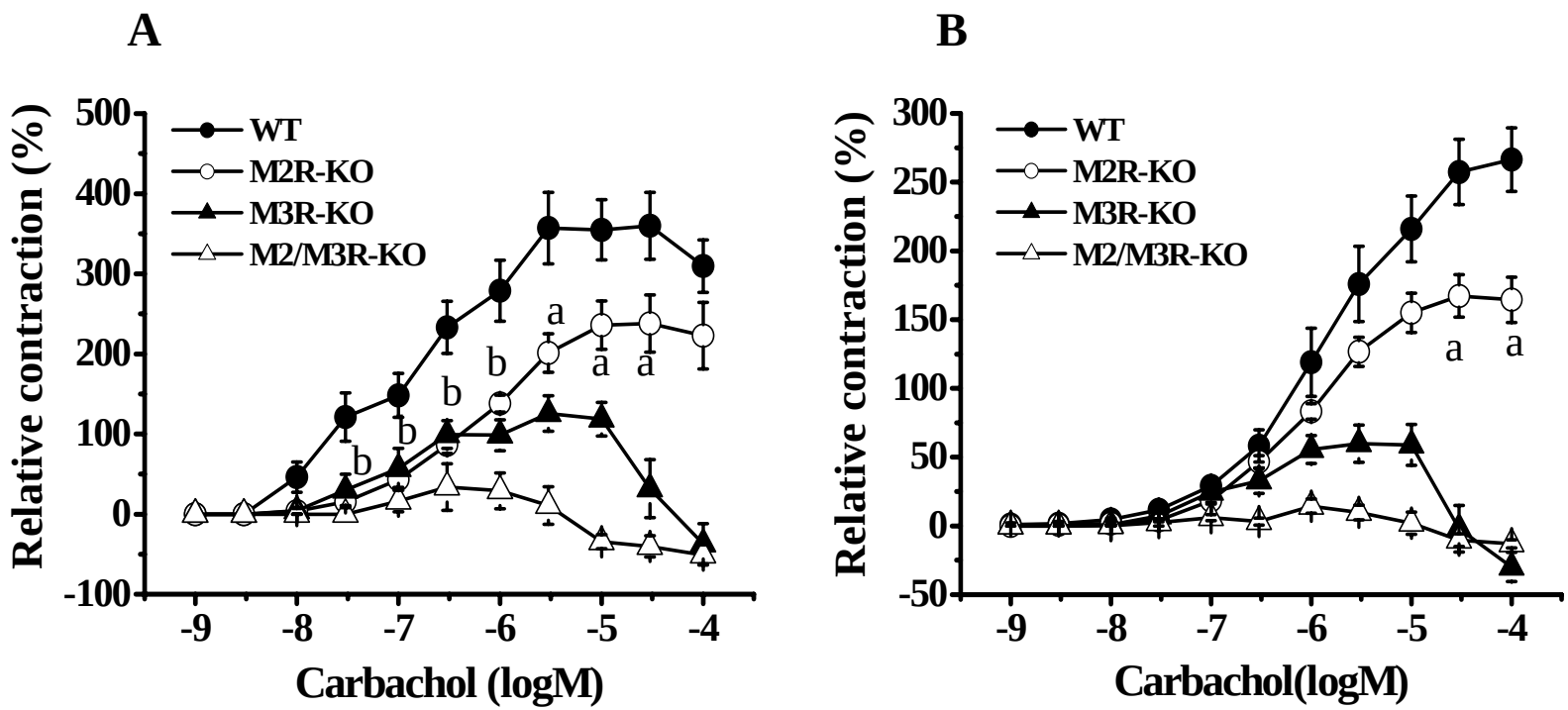

A
B
WT
M2R-KO
M3R-KO
M2/M3R-KO
Relative contraction (%)
Carbachol (logM)
Carbachol(logM)
a
b

Fig. 4

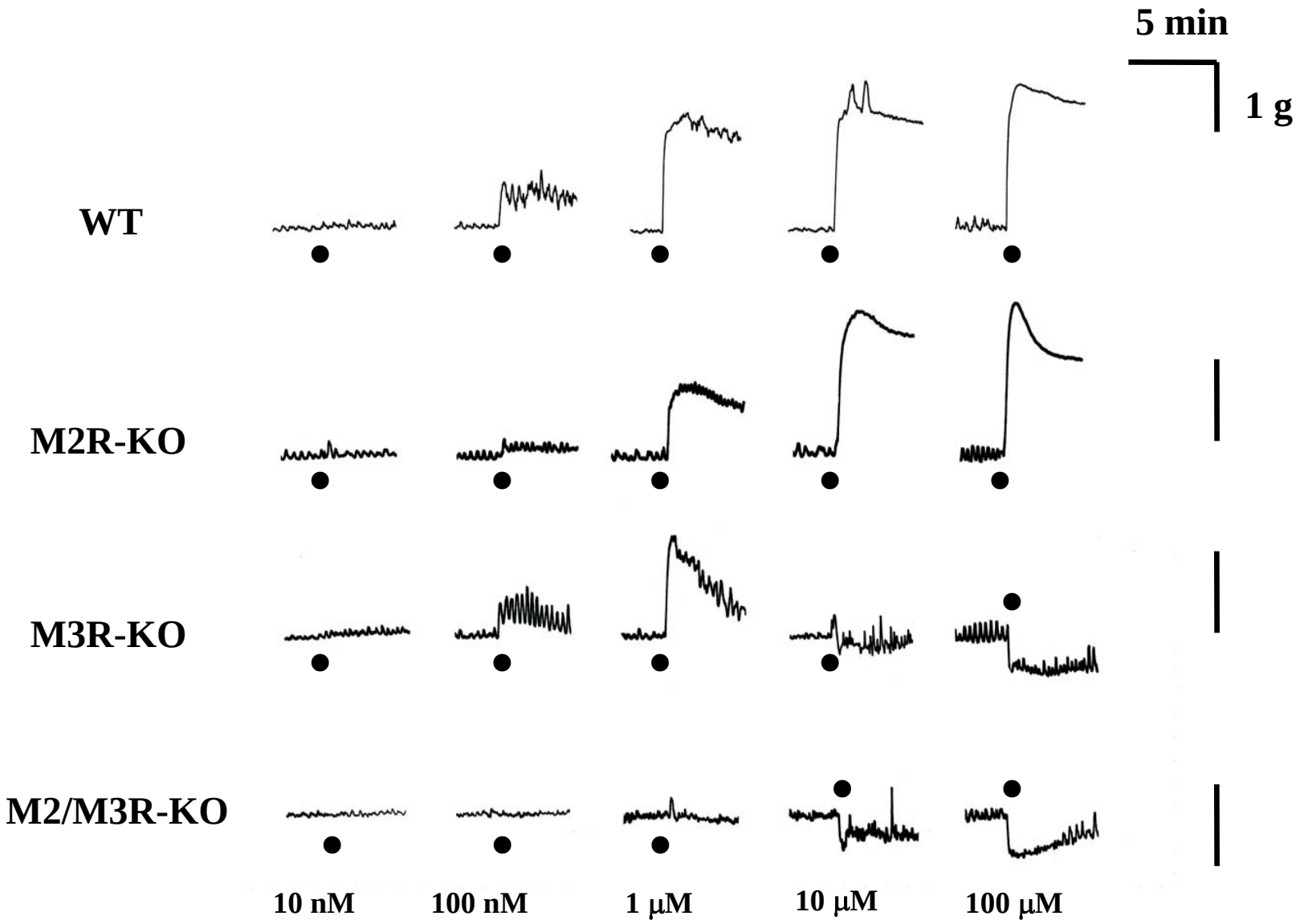
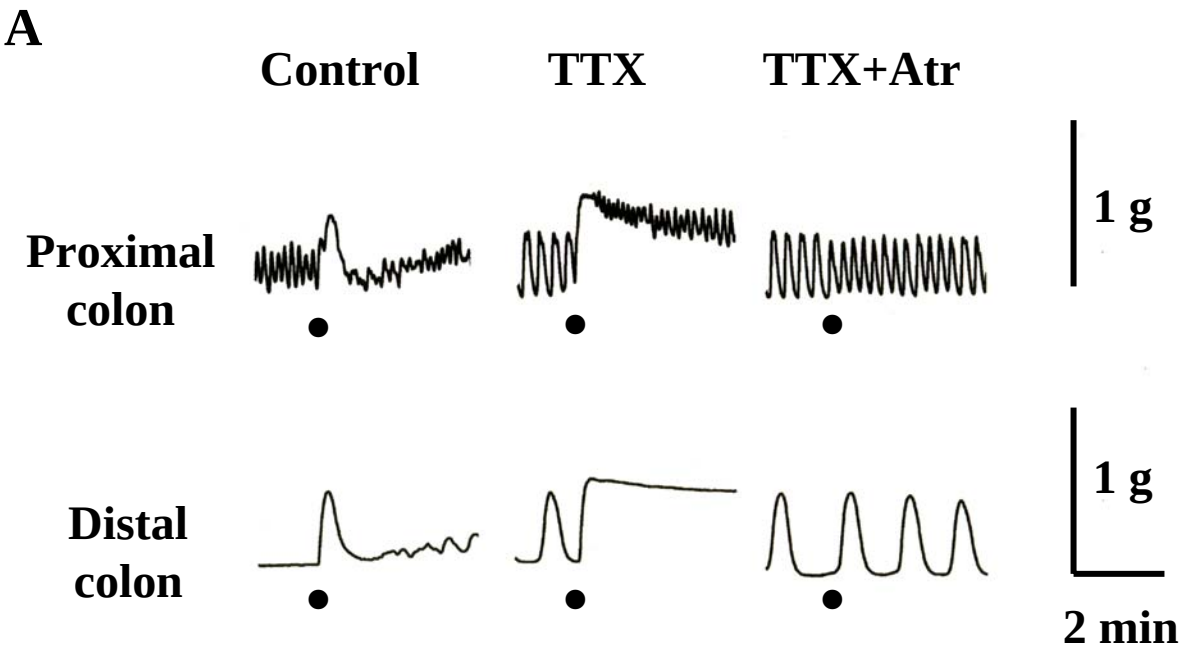


Fig.5


A
Control
TTX
TTX+Atr
Proximal colon
Distal colon
1 g
1 g
2 min

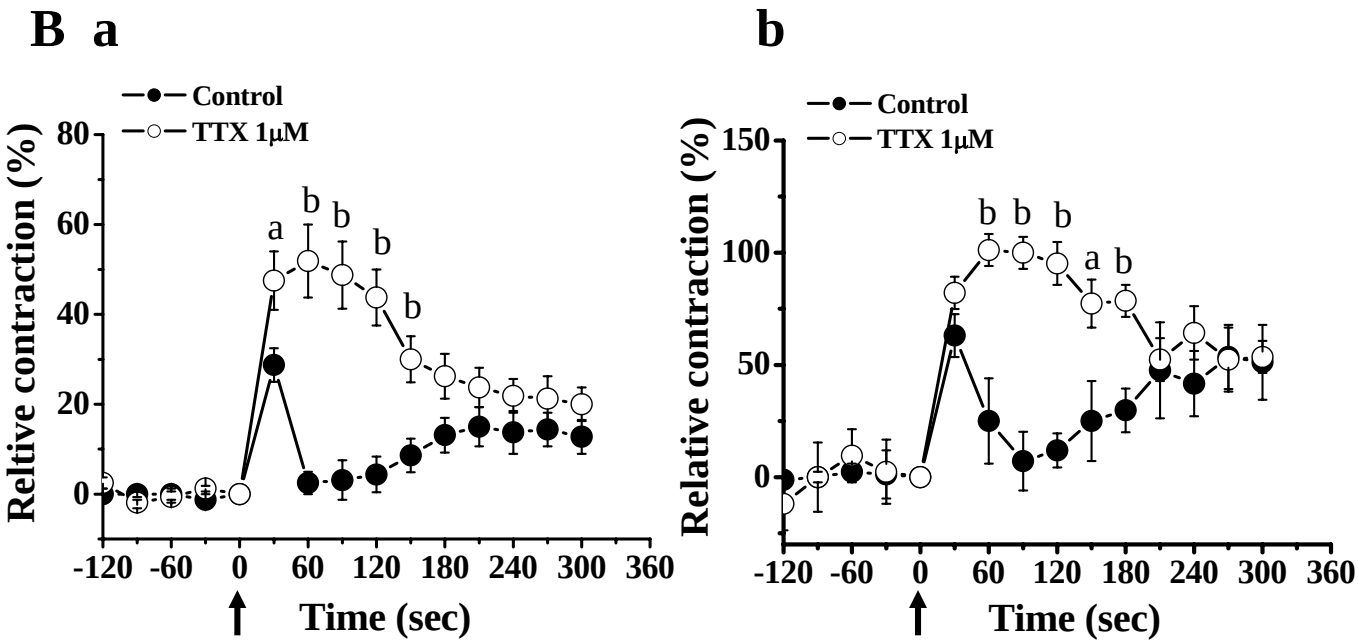
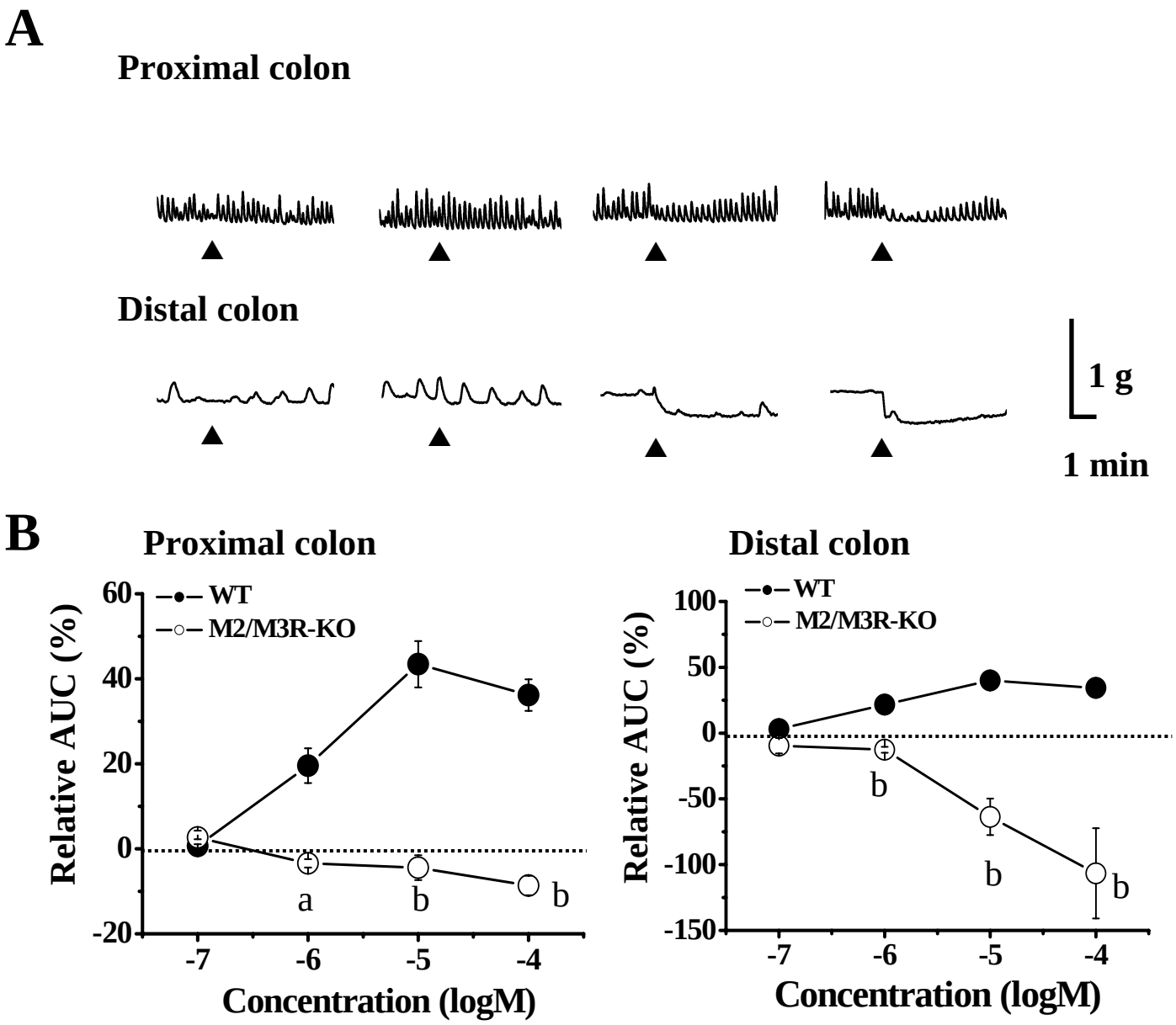

B a
Control
TTX 1μM
Reltive contraction (%)
80
60
40
20
0
a
b
b
b
b
-120
-60
0
60
120
180
240
300
360
Time (sec)
b
Control
TTX 1μM
Relative contraction (%)
150
100
50
0
b
b
b
a
b
-120
-60
0
60
120
180
240
300
360
Time (sec)

Fig. 6


A
Proximal colon
Distal colon
1 g
1 min
B
Proximal colon
Distal colon
WT
M2/M3R-KO
Relative AUC (%)
Concentration (logM)
a
b
b
b
b
b