Muscarinic M_2 acetylcholine receptor distribution in the guinea pig gastrointestinal tract

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Abstract

In the enteric nervous system, acetylcholine is the most common neurotransmitter to induce gastrointestinal smooth muscle contractions. Cholinergic signaling is mediated by muscarinic acetylcholine receptors on the surface of smooth muscle cells. Five different muscarinic receptor subtypes (M_1-M_5) have been identified and characterized, all of which belong to the superfamily of the G-protein-coupled receptor. The muscarinic M_2 acetylcholine receptor is the major muscarinic receptor subtype expressed by smooth muscle tissues in the gastrointestinal tract, where it is coexpressed with a smaller population of M_3 receptor. In this study, we examined the immunohistochemical distribution of the M_2 receptor using a specific antibody. M_2 receptor-like immunoreactivity (M_2R-LI) was mainly observed as associated with smooth muscle cells in the gastrointestinal tract. M_2R-LI in smooth muscle cells was distributed throughout the cell membrane associated with caveolae. In the proximal colon, M_2R-LI in the smooth muscle cells was weak. In the small intestine, interstitial cells of Cajal that possessed neurokinin 1 receptor-LI had intense M_2R-LI. In the proximal colon, intramuscular and myenteric interstitial cells of Cajal exhibited M_2R-LI. These findings indicate that, in the gastrointestinal musculature, M_2 receptors are distributed both in the smooth muscle cells and interstitial cells of Cajal, suggesting that the M_2 receptor

elicits smooth muscle cell contraction and the interstitial cells of Cajal are the sites of innervation by enteric cholinergic neurons.

Key words: enteric nervous system, smooth muscle, interstitial cells of Cajal, caveolae, acetylcholine, neurokinin 1 receptor

Introduction

Acetylcholine is distributed widely and acts as a neurotransmitter in the central nervous system and peripheral nervous system. In the enteric nervous system, acetylcholine is the most common neurotransmitter to induce gastrointestinal tract contractions. In the gastrointestinal tract, acetylcholine is released from excitatory enteric motor neurons that have choline acetyltransferase and vesicular acetylcholine transporter, and mediates immediate smooth muscle contraction (Bornstein et al., 2004; Furness, 2000). Acetylcholine is believed to be functionally predominant in inducing smooth muscle contractions.

Acetylcholine signaling is mediated by nicotinic acetylcholine receptors and muscarinic acetylcholine receptors on the cell surface. Muscarinic acetylcholine receptors are known to control of many central and peripheral cholinergic responses (Wess, 1996; Wess, 2004). To date, five different muscarinic receptor subtypes (M_1-M_5) have been identified and characterized (Caulfield and Birdsall, 1998; Wess, 1996), all of which belong to the superfamily of the G-protein-coupled receptor. M_1, M_3, and M_5 receptors act preferentially to activate the phospholipase C pathway through selective coupling to G protein Gq/G11, whereas M_2 and M_4 receptors mainly mediate the inhibition of adenylate cyclase by coupling to G protein Gi/Go (Caulfield and Birdsall, 1998; Wess, 1996). Many researches, including

immunohistochemistry and in situ hybridization studies, have shown that muscarinic receptors are present in many organs and tissues (Dorje et al., 1991; Eglen et al., 1996; Ehlert et al., 1997; Levey, 1993; Wess, 2004). The M_1 receptor is mainly distributed in the brain, M_2 receptor in the brain, heart and smooth muscle organs, M_3 receptor in the brain, smooth muscle organs, exocrine glands and eyes, M_4 receptor in the brain and lung, and M_5 receptor is expressed at rather low levels in both neuronal and nonneuronal cells. Because of the lack of ligands endowed with a high degree of receptor subtype selectivity and as most tissues express two or more muscarinic receptor subtypes, identification of the physiological and pathophysiological roles of the individual muscarinic receptor subtypes has proved difficult.

In smooth muscle tissues such as the gastrointestinal tract, urogenital tract and respiratory tract, the M_2 receptor is the major muscarinic receptor subtype, where it is coexpressed with a smaller population of M_3 receptors (Eglen et al., 1996; Ehlert et al., 1997; Levey, 1993). In the gastrointestinal tract, more than 70% muscarinic receptors are composed from the M_2 receptor and about 20% of muscarinic receptors are from the M_3 receptor. From a molecular study using the RT-PCR technique, both smooth muscle cells and interstitial cells of Cajal expressed both M_2 and M_3 receptors (Epperson et al., 2000). Interstitial cells of Cajal are the third cell types different from smooth muscle cells and enteric

neurons in the gastrointestinal musculature and are involved in muscle contraction and neuronal modulation of contraction (Rumessen and Vanderwinden, 2003; Sanders, 1998). To help decipher the biological function of muscarinic M_2 acetylcholine receptors in vivo, the histological and cytological distribution of M_2 receptor must be elucidated. In this study, we examined the immunohistochemical distribution of the M_2 receptor using a specific antibody, and the intracellular distribution of the M_2 receptor on the electron microscopic level.

Experimental procedures

Adult male Hartley guinea pigs aged 4-6 weeks and weighing 250-400 g (Japan SLC, Japan) were used in this study. The use and treatment of animals followed the Guidelines for Animal Experiments, University of Fukui Faculty of Medical Sciences. All guinea pigs were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg) before fixation.

Guinea pigs were transcardially perfused with Zamboni's fixative (Iino, 2000). The gastrointestinal tract was dissected out and further immersed in Zamboni's fixative for 4 h at room temperature. After washing with 0.01 M phosphate-buffered saline (PBS, pH 7.2), the specimens were soaked overnight in 30% sucrose in 0.1 M phosphate buffer (PB, pH 7.4) at 4°C, embedded in OCT compound (Miles, USA) and frozen quickly. Twelve-μm thick sections were cut using a cryostat and thaw-mounted onto poly-L-lysine-coated glass slides. The sections were treated with methanol containing 0.3% H_2O_2. After washing with PBS, sections were incubated for 1 h in 10% normal goat serum (diluted with PBS), and then reacted with rat anti-M_2 receptor (1:500 with PBS, MAB367, Chemicon, USA) (Levey et al., 1995a) for 12 h at room temperature. Sections were washed three times with PBS, and then reacted with biotinylated goat anti-rat IgG (1:200 with PBS, Vector, USA) for 1 h. After

washing with PBS, they were reacted with avidin-biotin-peroxidase complex (ABC Kit, Vector) for 1 h. The sections were incubated for several minutes with a solution containing 0.03% diaminobenzidine, 0.005% H_2O_2 in 0.1 M Tris-HCl, pH 7.6. All sections were dehydrated with ethanol and mounted in Entellan (Merck, Germany).

For fluorescence immunohistochemistry, sections were incubated in a mixture of rat anti-M_2 receptor (1:500 with PBS) and mouse anti-smooth muscle actin (1:600 with PBS, clone 1A4, Sigma, USA), mouse anti-actin (1:500 with PBS, clone B4, ICN, USA), rabbit anti-neurokinin 1 receptor (1:500 with PBS, #94168) (Grady et al., 1996; Southwell and Furness, 2001), or mouse anti-caveolin 1 (1:100 with PBS, clone 2297, Transduction Laboratories, USA). Then sections were incubated for 1 h with the biotinylated anti-rat IgG (1:200 with PBS, Vector), then for 1 h with FITC (fluorescein isothiocyanate)-conjugated streptoavidin (1:100 with PBS, Vector, USA) and Cy3-conjugated anti-mouse IgG (1:200 with PBS, Jackson, USA), Texas Red-conjugated anti-rabbit IgG (1:100 with PBS, Vector). These sections were examined with a confocal laser-scanning microscope GB200 (Olympus, Japan) with excitation wavelength of 488 nm and 543 nm. Immunohistochemical study using whole mount preparations was carried out by the method described previously (Iino et al., 2004).

For immunoelectron microscopy, three guinea pigs were transcardially perfused with Zamboni's fixative plus 0.1% glutaraldehyde. The intestine was dissected out and further immersed in Zamboni's fixative for 4 h at room temperature. Tissues treated as above were cut by cryostat at 12μm and mounted on poly-L-lysine coated slides. Specimens were immunostained as were those for light microscopic observations. After coloration in diaminobenzidine solution, specimens were post-fixed in 1% OsO_4 in PB for 1 h and block-stained with uranyl acetate, dehydrated in ethanol and embedded in Epok 812 (Oken, Japan). Ultrathin sections were examined without electron staining using an electron microscope H-7000 (Hitachi, Japan).

Anti-M_2 receptor rat monoclonal antibody was produced as previously reported and the specificity was checked by Levey et al. (1995ab). M_2 receptor knockout mice showed no specific immunoreactivity for this antibody (Takeuchi et al., 2005). Specificity of this immunohistochemical study was checked by unimmunized rat serum instead of the anti-M_2 receptor antibody, and no specific immunoreactivity was observed.

Results

M_2 receptor-like immunoreactivity (M_2R-LI) was observed associated with smooth muscle cells in the gastrointestinal tract (Fig. 1, Table 1). The external muscle layers, except the esophagus, were immunoreactive for M_2 receptor. The intensity of immunoreactivity was higher in the stomach, small intestine, cecum, distal colon and rectum than in the proximal colon. Distinct immunoreaction was also observed in the muscularis mucosae in the pylorus, colon and rectum.

1, Esophagus

In the esophagus, both external muscle layer and muscularis mucosae showed no M_2R-LI (Fig. 1). In the lower esophageal sphincter (Fig. 2A), M_2R-LI was observed only in the smooth muscle cells. The enteric ganglia and interstitial cells of Cajal in the esophagus were free from reaction.

2, Stomach

In the gastric fundus and corpus (Figs. 1B, 2B), M_2R-LI was clearly observed in the musculature. In the circular muscle, most smooth muscle cells, except a few cells associated with submucosa, showed distinct immunoreactivity at their surface. In the submucosal border, M_2R-LI in the smooth muscle cells was low or absent. In the longitudinal muscle,

all smooth muscle cells showed M_2R-LI as in circular layer cells. The enteric ganglia and interstitial cells of Cajal were free from reaction. In the pylorus (Fig. 1C), although the circular muscle had distinct immunoreactivity as observed in the fundus and corpus, the longitudinal muscle showed weak M_2R-LI. The muscularis mucosae in the fundus and corpus had no immunoreactivity (Fig. 1B), whereas that in the pylorus had intense M_2R-LI (Fig. 1C).

3, Small intestine

In the duodenum (Fig. 1D), jejunum (Fig. 1E) and ileum, almost all smooth muscle cells in the musculature had M_2R-LI on their surface. Using double fluorescence immunohistochemistry with anti-M_2 receptor and anti-actin antibodies (Figs. 3A-F), all smooth muscle cells in the outer circular muscle and longitudinal muscle had distinct M_2R-LI. The inner circular muscle, localized between the outer circular muscle and the submucosa, had no M_2R-LI.

In the deep muscular plexus layer (DMP) between the inner and outer circular muscle (Figs. 3D-F), there were a small number of M_2R-LI cells that were not stained by actin antibodies. Different from the smooth muscle cells, these cells had M_2R-LI in the periphery and cytoplasm. These cells showed neurokinin 1 receptor-LI (Figs. 3G-I), which is a marker

of the interstitial cells of Cajal in the DMP (Iino et al., 2004; Lavin et al., 1998; Southwell and Furness, 2001). Though, interstitial cells of Cajal in the small intestine are also distributed in the myenteric layer and interstitial cells of Cajal in the duodenum and proximal jejunum show neurokinin 1 receptor-LI (Lavin et al., 1998; Rumessen and Vanderwinden, 2003), there were no specific cells having M_2R-LI in the myenteric layer.

In immunoelectron microscopy (Fig. 4), M_2R-LI was detected in smooth muscle cells in the outer circular muscle, longitudinal muscle and interstitial cells of Cajal in the DMP. The smooth muscle cells in the inner circular muscle were flattened and much smaller than the smooth muscle cells in the outer circular muscle cells, and completely free from M_2R-LI (Figs. 4A-C). The interstitial cells of Cajal in the DMP showed intense immunoreaction in the cell surface and cytoplasm (Figs. 4A-D). At an ultrastructural level, interstitial cells of Cajal in the DMP had conspicuous mitochondria, smooth endoplasmic reticulum and Golgi apparatus, relatively sparse rough endoplasmic reticulum, and myofilaments. Many caveolae were observed lining the plasma membrane. These structures are consistent with the previously described characteristics of interstitial cells of Cajal in the DMP (Komuro, 1999; Rumessen and Vanderwinden, 2003). Cytoplasmic processes of the interstitial cells of Cajal in the DMP had intense M_2R-LI (Figs. 4A-D). These cells had gap junctions with

themselves (Fig. 4C) or with the smooth muscle cells in the outer circular layer (Fig. 4D).

M_2R-LI in the smooth muscle cells was distributed throughout the cell membrane at the cytoplasmic surface in the electron microscopic level. These immunoreactivities were detected as a patchy pattern and most reactions were around or associated with caveolae (Fig. 4F). Using double fluorescence immunohistochemistry with anti-M_2 receptor (Fig. 3J) and anti-caveolin 1 antibodies (Fig. 3B), M_2R-LI was colocalized with caveolin 1-LI.

4, Large intestine

In the cecum (Fig. 1F), both circular and longitudinal muscle cells showed distinct M_2R-LI. The muscularis mucosae of the cecum had no M_2R-LI.

In the proximal colon (Figs. 1G, 5A-E), M_2R-LI in smooth muscle cells that showed smooth muscle actin-LI was weak. The smooth muscle cells in the inner fourth of the circular muscle had no M_2R-LI and those in the outer circular muscle and longitudinal muscle had weak immunoreactivity at their surface. In the circular and longitudinal muscle layers, there were several intense immunopositive cells. These cells were situated at the interstitial space among the smooth muscle cells and had a bipolar or multipolar shape with long processes along the smooth muscle cells. Using double fluorescence immunohistochemistry with anti-M_2 receptor (Fig. 5A) and anti-actin antibodies (Fig. 5B), these cells with intense

M_2R-LI showed no actin-LI. Using whole mount preparations, M_2R-LI cells in the circular and longitudinal muscle (Figs. 5CD) showed bipolar or multipolar shape with long slender processes. In the myenteric layer, there was another cell type with M_2R-LI (Figs. 5ABE). These cells had no actin-LI, were situated around the myenteric ganglia and showed multipolar shape.

Using immunoelectron microscopy in the proximal colon, intense M_2R-LI was detected in cells distributed both in the circular and longitudinal muscle layers (Figs. 6ABEF). These cells possessed oval nuclei with scant perinuclear cytoplasm in comparison to smooth muscle cells and had many mitochondria. These cells resembled the interstitial cells of Cajal in the muscle layer of the proximal colon (Komuro, 1999; Rumessen and Vanderwinden, 2003). Around the myenteric ganglia, M_2R-LI cells had long processes and many mitochondria (Figs. 6CD). These characteristics show M_2R-LI cells as interstitial cells of Cajal in the myenteric layer. The smooth muscle cells in the musculature had weak immunoreactivity around the caveolae.

In the distal colon and rectum (Figs. 1HI), most smooth muscle cells in the musculature had distinct M_2R-LI as observed in the stomach and small intestine. The inner surface of the circular muscle layer showed weak immunoreactivity. Using double

immunohistochemistry with anti-M_2 receptor (Fig. 5F) and anti-actin antibodies (Fig. 5G), we could clearly observe that most smooth muscle cells had M_2R-LI at their surfaces. In the interstitial space of the muscle layer and around the myenteric ganglia, there were no M_2R-LI interstitial cells of Cajal as observed in the proximal colon. In the muscularis mucosae of the colon and rectum, only longitudinal arranged smooth muscle cells had M_2R-LI at their surface. The circular smooth muscle cells in the muscularis mucosae were free from reaction.

Discussion

In this study, we obtained evidence that muscarinic M_2 acetylcholine receptor-like immunoreactivity (M_2R-LI) is distributed in the smooth muscle cells and interstitial cells of Cajal in the musculature of the guinea pig gastrointestinal tract. These findings are the first evidence of M_2 receptor distribution on a morphological level.

Presence of M_2 receptor in the smooth muscle tissue is known by immunoprecipitation studies, ligand binding studies and PCR studies (Dorje et al., 1991; Eglen et al., 1996; Ehlert et al., 1997; Epperson et al., 2000; Levey, 1993; So et al., 2003). There is only report on the tissue level of M_2 receptor distribution by Takeuchi et al. (2005). They showed M_2 receptor distribution in the murine ileal smooth muscle using same antibody with our study. Using double fluorescence immunohistochemistry and immunoelectron microscopy, we showed clearly M_2R-LI in the smooth muscle cells. The M_2 receptor is the predominant muscarinic receptor subtype expressed by the gastrointestinal tract, where it is coexpressed with a smaller population of M_3 receptor (Eglen et al., 1996; Ehlert et al., 1997; Levey, 1993). In vitro experiment using M_2 receptor knockout mice showed that carbachol was two-fold more potent in wild-type stomach fundus than M_2 receptor knockout fundus (Stengel et al., 2000). Although carbachol-induced contractions were almost completely

abolished in the ileal muscle from mice deficient in both M_2 and M_3 receptors (Matsui et al., 2002), M_3 receptor knockout mice studies showed that the carbachol-induced contraction of ileal and fundus smooth muscle tissues was reduced by 50 - 70% (Matsui et al., 2000, Stengel et al., 2002). These studies suggest that M_2 receptor contributes to the efficiency of muscarinic agonist-induced smooth muscle contraction. From our observation, almost all gastrointestinal smooth muscle cells showed M_2R-LI, except weakly immunopositive smooth muscle cells in the proximal colon. These morphological findings support the abundant and functional expression of M_2 receptor in the gastrointestinal tract. Our observation also revealed that smooth muscle cells in the gastrointestinal tract expressed different levels of M_2 receptors on the immunohistochemical level. For example, smooth muscle cells in the inner parts of the stomach, small intestine and colon showed no or weak immunoreactivity and smooth muscle cells of the muscularis mucosae in the stomach and small intestine showed no M_2R-LI. These results suggest that there are different sensitivities for muscarinic stimulation in the gastrointestinal smooth muscle cells. We need to examine other types of muscarinic receptors such as M_3 and M_4 receptors.

In the colon, the muscarinic receptor subtype expressions are similar to those seen in other gastrointestinal tracts, however, the expression populations of M_2 receptors and M_3

receptors are different between humans and rats (Eglen et al., 1996). In the rat colon, lower proportions of M_2 receptor, ranging from 39% to 55%, have been reported (Gomez et al., 1992; Zhang, 1996). Our findings show that in the proximal colon, although the smooth muscle cells contain less M_2 receptor, the interstitial cells of Cajal contain significant M_2 receptor. These specific distributions suggest that the proximal colon showed different muscarinic receptor subtype populations to those seen in other gastrointestinal tracts.

Caveolae are small flask-like-shaped invaginations of the plasma membrane and are observed on muscle cells, endothelial cells and so on. A number of G-protein-coupled receptors including the B2 bradykinin receptor, β-adrenergic receptor, cholecystokinin receptor, endothelin receptor, and angiotensin II receptor have been shown to be located within caveolae in native conditions or upon agonist stimulation (Ostrom and Insel, 2004; Razani et al., 2002). There are many molecules crucial for transducting the signals initiated by receptors, e.g., G proteins, adenylate cyclase, protein kinase C and components of the mitogen-activated protein (MAP) kinase cascade (Ostrom, 2002; Ostrom and Insel, 2004). Therefore, caveolae appear to act as centers that concentrate certain signaling molecules. In this study, we observed M_2R-LI around the smooth muscle caveolae in native conditions. Taken together, these findings suggest that smooth muscle caveolae are involved in M_2

receptor signaling via G protein Gi/Go.

Regulated intracellular trafficking after stimulation controls the activity and cell surface expression of G-protein coupled receptors such as muscarinic receptors. In cardiac myocytes, a large fraction of sarcolemmal M_2 receptor is translated into caveolae upon agonist carbachol binding (Feron et al., 1997). The internalization of G-protein-coupled receptors from the cell surface is a commonly observed phenomenon following agonist-stimulation (Ferguson, 2001). The activated M_2 receptors were sequestered into specialized intracellular compartments after agonist-stimulation in the expression systems of cultured cells (Delaney et al., 2002; Roseberry and Hosey, 1999). On the smooth muscle surface, neurokinin 1 receptors (substance P receptors), one of the G-protein-coupled receptors, are internalized after substance P stimulation (Southwell and Furness, 2001). We need to study internalization experiments to confirm the functional expression of M_2 receptor on smooth muscle cells.

Interstitial cells of Cajal are involved in muscle contraction and neuronal modulation of muscle contraction (Rumessen and Vanderwinden, 2003; Sanders, 1998; Ward and Sanders, 2001). Interstitial cells of Cajal express the receptor tyrosine kinase Kit and are identified by the Kit antibody (Rumessen and Vanderwinden, 2003). Although many researchers use rat

monoclonal antibody ACK2 to identify interstitial cells of Cajal (Beckett et al., 2002; Iino, 2000; Iino et al., 2004; Ward et al., 2000), we could not perform double fluorescence immunohistochemistry with Kit ACK2 and M_2 receptor because both antibodies were raised from a single species, rat. Therefore, we examined and identified interstitial cells of Cajal using other marker and experiments, such as electron microscope and whole mount preparations. In the small intestine, interstitial cells of Cajal in the DMP have neurokinin 1 receptor (Iino et al., 2004; Lavin, 1998; Rumessen and Vanderwinden, 2003), and we clearly observed that neurokinin 1 receptor-LI cells also had M_2R-LI. In an electron microscopic study, M_2 receptor-expressing cells exhibited many mitochondria, smooth endoplasmic reticulum and Golgi apparatus, caveolae and gap junctions. These features showed that M_2R-LI cells were interstitial cells of Cajal in the DMP (Komuro, 1999). In the proximal colon, we observed M_2R-LI cells having multipolar shape in the musculature using whole mount preparations. These cells showed oval nuclei with scant perinuclear cytoplasm in comparison to smooth muscle cells and had many mitochondria. Therefore, these cells are interstitial cells of Cajal in the muscle layer and myenteric layer in the proximal colon (Komuro, 1999).

Interstitial cells of Cajal are closely associated with varicose nerve terminals of

cholinergic neurons revealed by vesicular acetylcholine transporter (Beckett et al., 2002; Wang et al., 2000; Ward et al., 2000). Recent studies have suggested that interstitial cells of Cajal play an important role in neurotransmission (Ward and Sanders, 2001). In the murine small intestine, muscarinic stimulation using electrical field stimulation or acetylcholine administration caused protein kinase C-ε translocation in interstitial cells of Cajal in DMP (Wang et al., 2003). The translocation in interstitial cells of Cajal was blocked by tetrodotoxin or atropine, suggesting that these responses were due to acetylcholine release from nerve terminals and the activation of muscarinic receptors on interstitial cells of Cajal. There are several mice strains such as W/W^{v} and Sl/Sl^{d} in which interstitial cells of Cajal are deficient in specific parts of the gastrointestinal tract (Rumessen and Vanderwinden, 2003; Ward and Sanders, 2001). These mice lack interstitial cells of Cajal in the stomach fundus and showed significant reduction of neural responses despite the existence of normal cholinergic nerves (Beckett et al., 2002; Ward et al., 2000). These studies suggested that interstitial cells of Cajal are the primary sites of innervation by enteric cholinergic neurons. Taken together with our findings, interstitial cells of Cajal are involved in muscarinic neurotransmission via at least the M_2 receptor in the gastrointestinal musculature.

In summary, we examined the immunohistochemical distribution of the muscarinic

M_2 acetylcholine receptor, which is the major muscarinic receptor subtype expressed by smooth muscle tissues in the guinea pig gastrointestinal tract. M_2R-LI was mainly observed as associated with smooth muscle cells in the gastrointestinal tract. M_2R-LI in smooth muscle cells was distributed throughout the cell membrane associated with caveolae. Interstitial cells of Cajal in the small intestine deep muscular plexus and in the proximal colon had M_2R-LI. These findings indicate that M_2 receptors are distributed both in the smooth muscle cells and interstitial cells of Cajal, and suggest M_2 receptor mediated smooth muscle contraction and enteric cholinergic innervation to the interstitial cells of Cajal.

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Figure legends

Fig. 1 Distribution of M_2 receptor-like immunoreactivity (M_2R-LI) in the guinea pig gastrointestinal tract

A: Esophagus (Eso). There are no immunopositive structures. B: Gastric corpus (Cor). The external muscle layer shows intense M_2R-LI whereas the muscularis mucosae (arrowhead) show no reactivity. C: Gastric pylorus (Pyl). D: Duodenum (Duo). E: Jejunum (Jej). F: Cecum (Cec). Both inner and outer muscle layers show M_2R-LI. G: Proximal colon (PCo). M_2R-LI in the external muscle layer is weak without several cells. H: Distal colon (DCo). I: Rectum (Rec).

M: Mucosa, arrowheads: muscularis mucosae

Bar: 100 μm

Fig. 2 Distribution of M_2R-LI in the lower esophageal sphincter and stomach

A: Lower esophageal sphincter (LES). Arrowheads show immunopositive smooth muscle cells. Asterisks show immunonegative striated muscle cells.

B: Gastric corpus (Cor). Arrows show immunopositive smooth muscle cells. M_2R-LI is

distributed along the cell surface. Asterisks show immunonegative smooth muscle cells along the submucosa. These cells are free from immunoreaction.

CM: Circular muscle, LM: Longitudinal muscle

Bars: 100μm (A), 50μm (B)

Fig. 3 Distribution of M_2R-LI in the jejunum.

A and B are double-stained specimens using the anti-M_2 receptor antibody (green, M_2) and anti-smooth muscle actin antibody (red, Actin), respectively, and C is a merged image. Arrows show an inner circular muscle with no M_2R-LI and intense actin-LI. Arrowheads show muscularis mucosae with only actin-LI. CM (circular muscle) shows both immunoreactivities for M_2 and actin. ggl: Myenteric ganglion. D and E are double-stained specimens using the anti-M_2 receptor antibody (green, M_2) and anti-actin antibody (red, Actin), respectively, and F is a merged image. Asterisks show M_2R-LI cells that are negative for actin-LI situated between the inner and outer circular muscles. Arrows show an inner circular muscle with only actin-LI. CM (outer circular muscle) shows immunoreactivity for both M_2 and actin. G and H are double-stained specimens using the anti-M_2 receptor antibody (green, M_2) and anti-neurokinin 1 receptor antibody (red, NK1R),

respectively, and I is a merged image. Asterisks show cells with both M_2R-LI and neurokinin 1 receptor-LI. J and K are double-stained specimens using the anti-M_2 receptor antibody (green, M_2) and anti-caveolin 1 antibody (red, CV), respectively, and L is a merged image. Arrowheads show double immunopositive structures in the outer circular muscle. Bars: 10μm (A-I), 5μm (J-L)

Fig. 4 Electron microscopic demonstrations of M_2R-LI in the jejunum

A: M_2R-LI in the circular muscle. Intense immunoreactivity was observed in cell (asterisk) conceivable to the interstitial cells of Cajal between the inner circular muscle (ICM) and the outer circular muscle (OCM). The outer circular muscle cells have immunoreactivity, but the inner circular muscle cells show no reactivity. B: Immunopositive interstitial cells of Cajal shown in the asterisk (A) have a thin process. There are immunopositive cell processes (arrows) between inner (ICM) and outer circular muscle (OCM). Most immunopositive products are distributed around the cell periphery. C: Gap junctions (arrows) between the processes of immunopositive interstitial cells of Cajal (asterisks). These processes are between inner (ICM) and outer circular muscle (OCM). D: Gap junctions (arrows) between the interstitial cells of Cajal processes (asterisks) and the outer

circular muscle (OCM). N shows nerve bundles in the deep muscular plexus. E: Outer circular muscle. M_2R-LI (white arrowheads) is observed at the surface of the smooth muscle cells. F: High magnification micrograph of the outer circular muscle cells. Most immunoreactivity is observed associated with caveolae (white arrowheads).

Bars: 2 μm (AE), 1 μm (BCDF)

Fig. 5 Distribution of M_2R-LI in the colon.

A and B are double-stained proximal colon (PCo) using the anti-M_2 receptor antibody (M_2) and anti-actin antibody (Actin), respectively. Small arrows show M_2R-LI (A) and actin immunonegative (B) cells. These cells are situated at the interstitial space among the smooth muscles. Large arrows show immunopositive cells surrounding a myenteric ganglion (ggl). In the circular muscle (CM) and longitudinal muscle (LM), all smooth muscle cells shown with actin-LI have weak M_2R-LI. The inner fourth of the circular muscle has no M_2R-LI. C shows immunopositive cells (arrows) in the circular muscle. D shows immunopositive cells (arrows) having multipolar shape in the myenteric layer. E shows multipolar immunopositive cells (arrows) in the longitudinal muscle. F and G are double-stained distal colon (DCo) using the anti-M_2 receptor antibody (M_2) and anti-actin

antibody (Actin), respectively. All smooth muscle cells in the circular (CM) and longitudinal (LM) musculature show intense M_2R-LI. In the muscularis mucosae, though longitudinal smooth muscle cells (arrowheads) have intense M_2R-LI, circular smooth muscle cells have no M_2R-LI.

Bars: 10μm

Fig. 6 Electron microscopic demonstration of M_2R-LI in the proximal colon

AB: Immunopositive cells (asterisks) in the circular muscle. Nerve bundle (N) is situated near the immunopositive cells. CD: Immunopositive cell (asterisk) between the circular (CM) and longitudinal (LM) muscles (C), or between the circular muscle and the myenteric ganglion (G) (D). These cells have large oval nuclei with scant perinuclear cytoplasm and long thin processes. EF: M_2R-LI cells (asterisks) in the longitudinal muscle. The immunopositive cell has a large oval nucleus with scant perinuclear cytoplasm and long process.

Bars: 2 μm (ACDE), 1 μm (BF)

Table 1. M_2 receptor-like immunoreactivity in the guinea pig gastrointestinal tract

	Smooth muscle in the external muscle	Interstitial cells of Cajal	Smooth muscle in the muscularis mucosae
Esophagus	-	-	-
Fundus corpus	++	-	-
Pylorus	++	-	+
Small intestine	++	++ (DMP) -(MY)	-
Cecum	++	-	-
Proximal colon	+	++ (IM, MY)	++ (L) - (C)
Distal colon	++	-	++ (L) - (C)
Rectum	++	-	++ (L) - (C)

++: intense immunopositive, +: immunopositive, -: immunonegative

DMP: deep muscular plexus layer, IM: intramuscular, MY: myenteric, L: longitudinal, C: circular

Fig.1

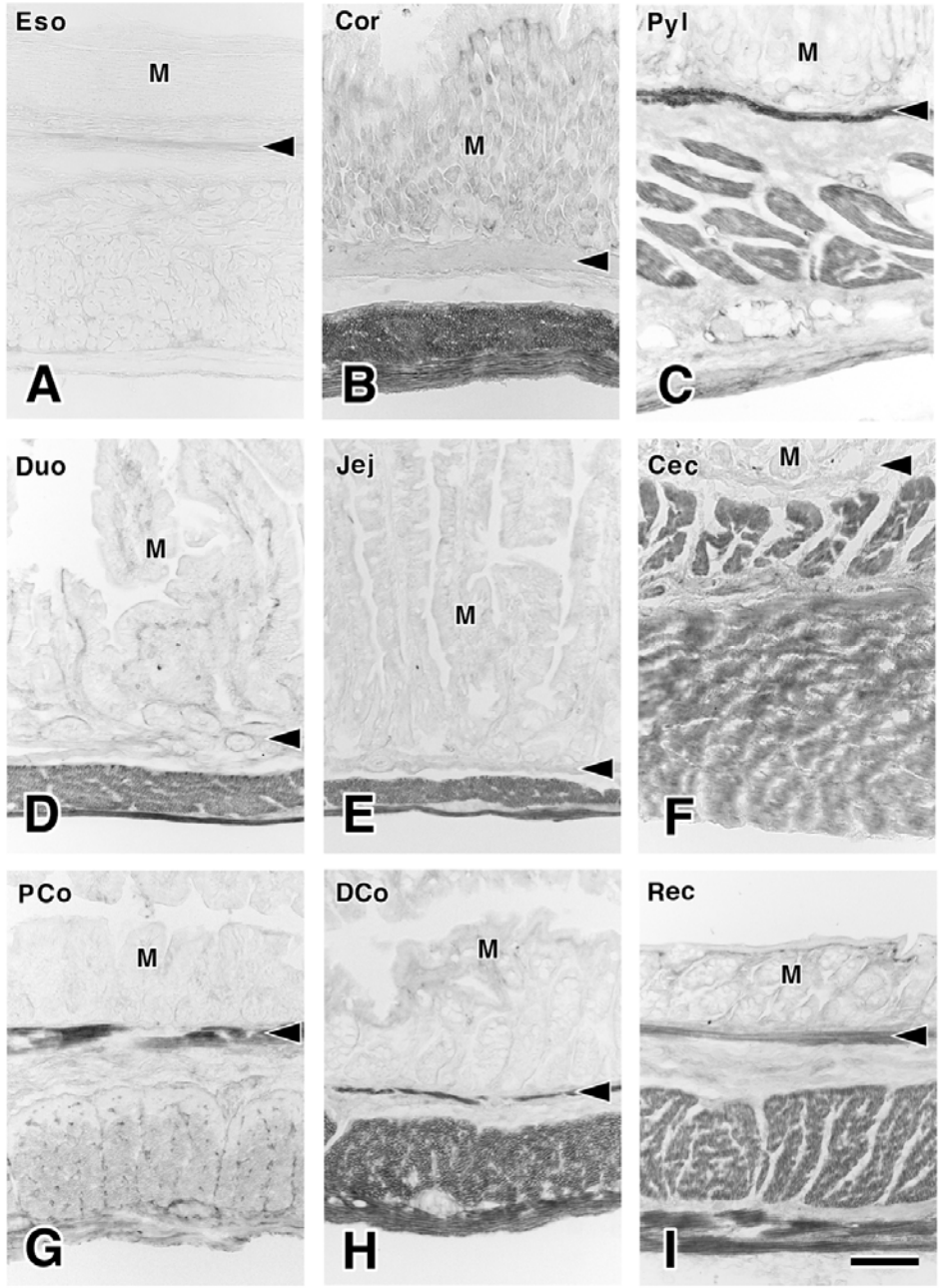
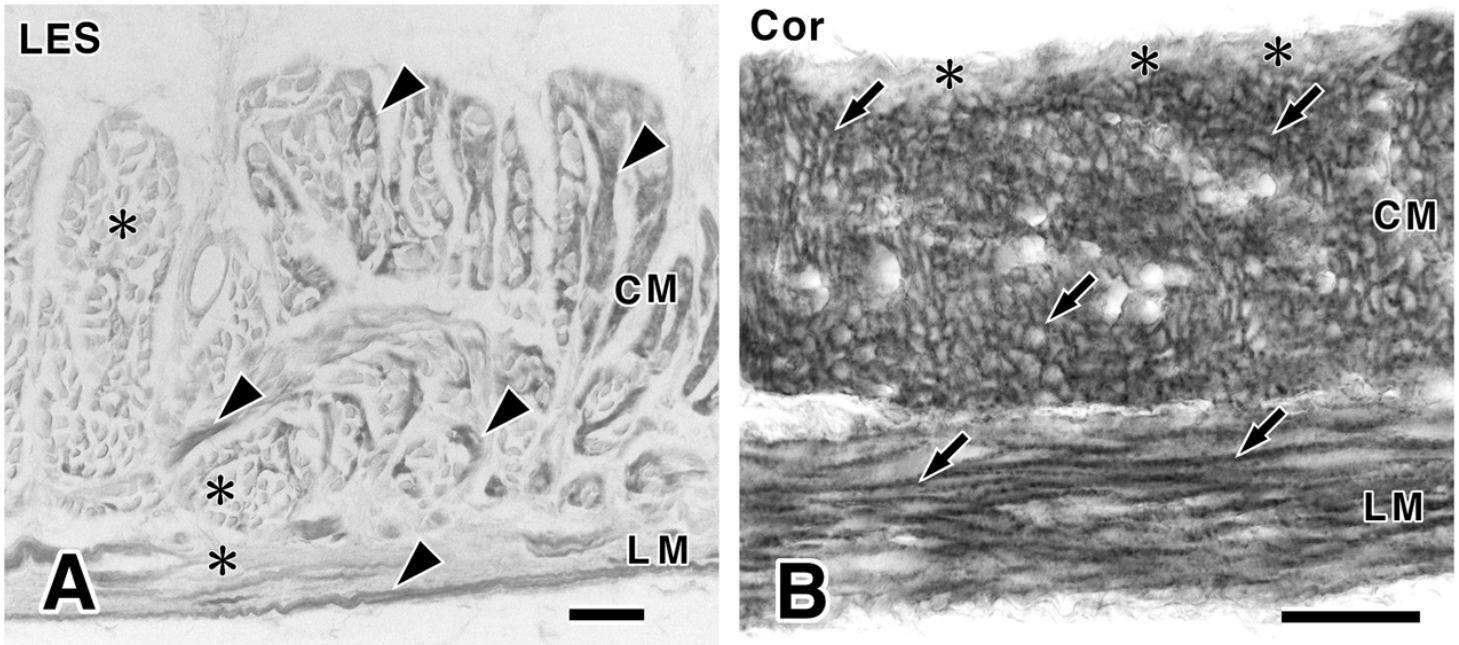


Fig.2



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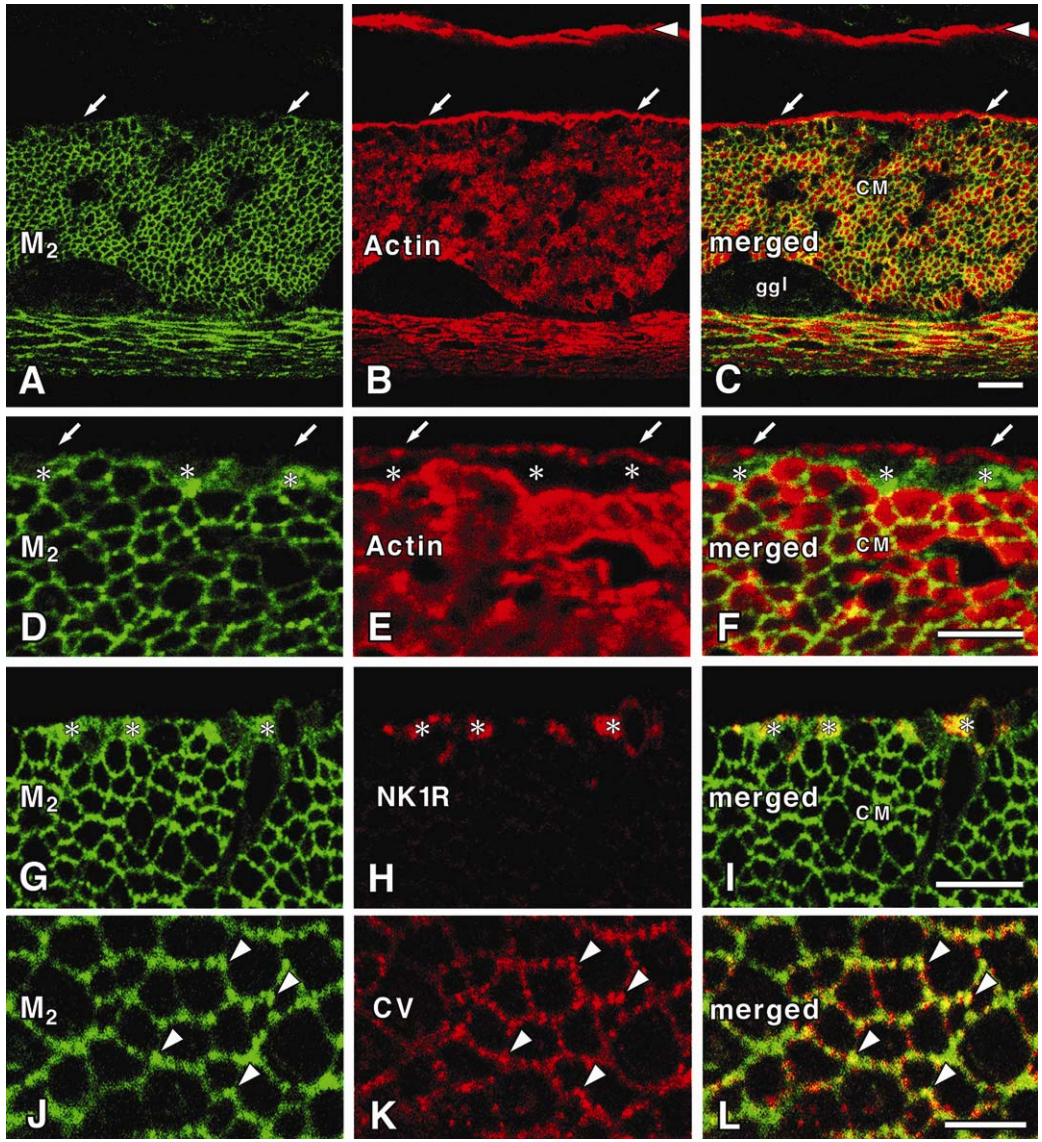
M2
A
Actin
B
CM
merged
ggl
C
M2
D
Actin
E
merged
CM
F
M2
G
NK1R
H
merged
CM
I
M2
J
CV
K
merged
L

Fig.4

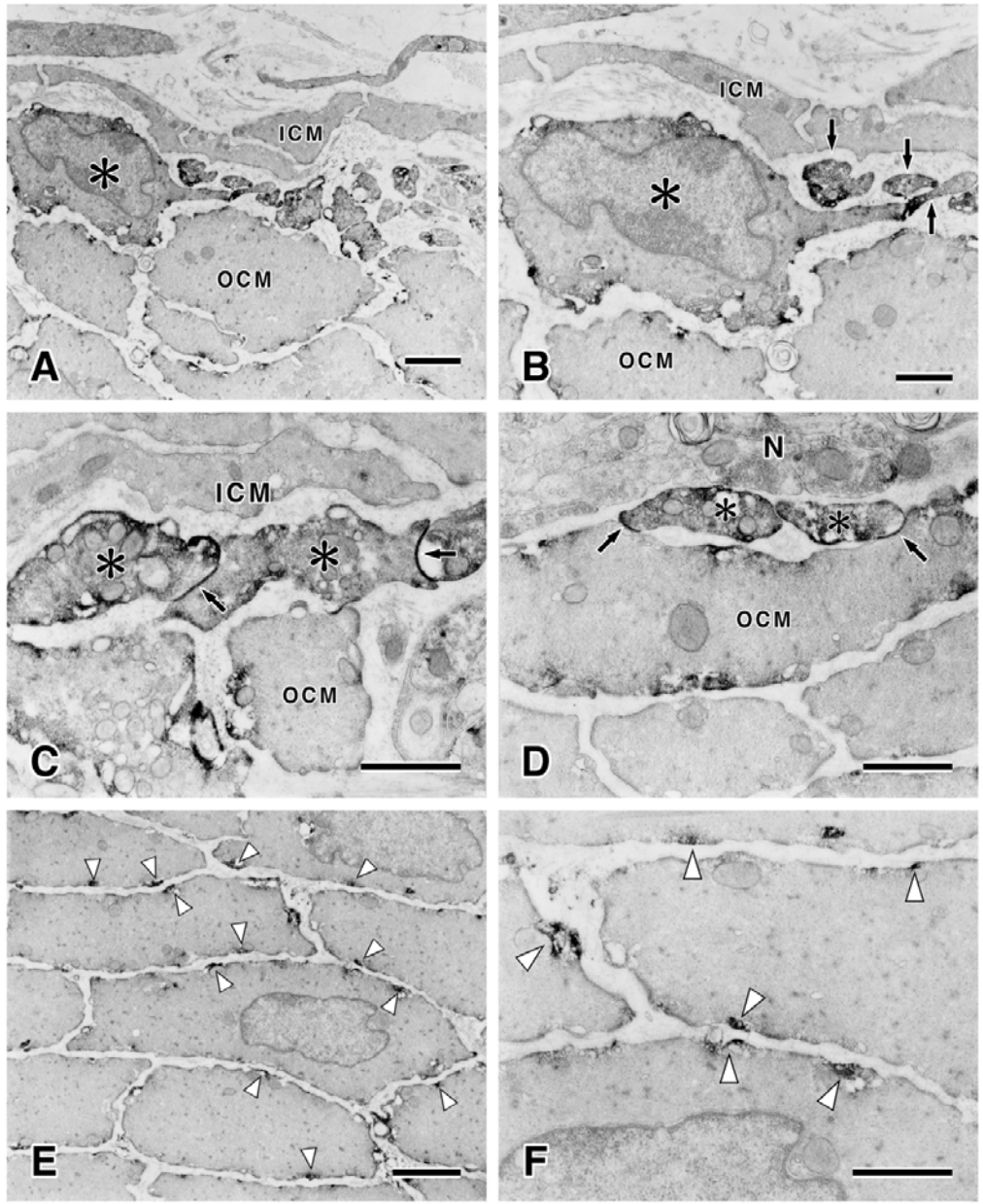

Fig.5

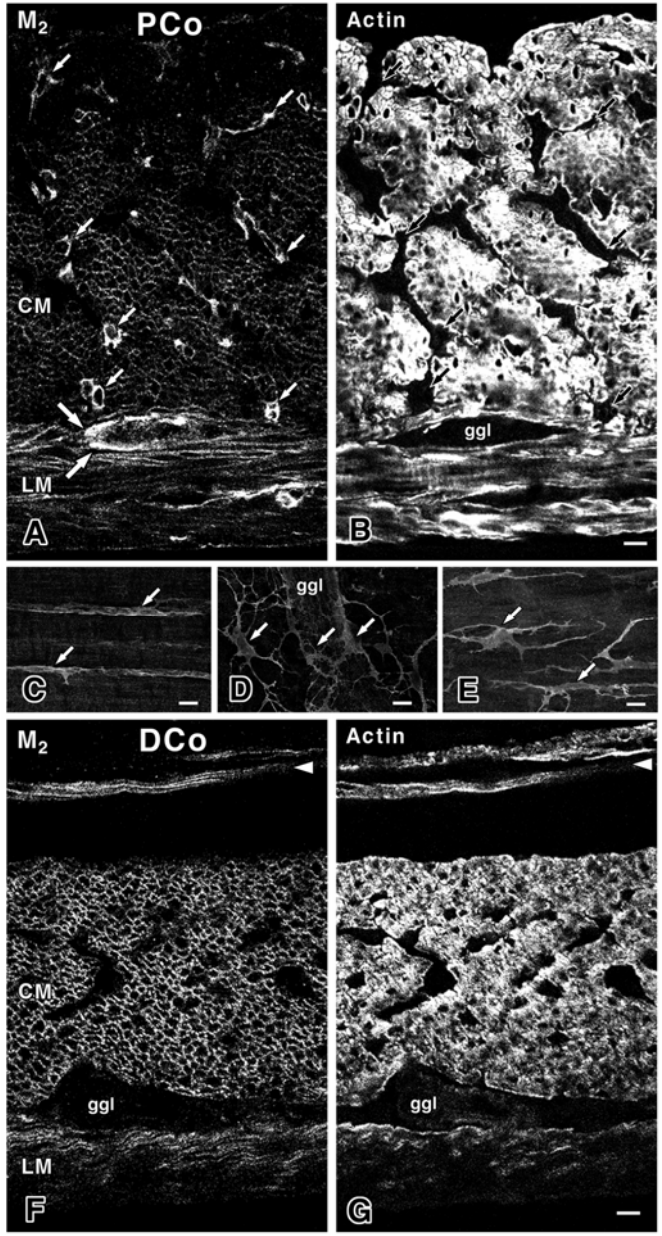

Fig.6