Localization of TRPV1 and contractile effect of capsaicin in mouse large intestine: high abundance and sensitivity in rectum and distal colon

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1Laboratory of Pharmacology, Faculty of Pharmaceutical Sciences, Josai International University, Togane, Chiba; 2Department of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, Chiba University, Image-ka, Chiba, Japan; and 3Neuroscience Centre, Institute of Cell and Molecular Science, Barts and The London School of Medicine and Dentistry, Whitechapel, London, United Kingdom

Submitted 5 October 2008; accepted in final form 28 May 2009

Matsumoto K, Kurosawa E, Terui H, Hosoya T, Tashima K, Murayama T, Priestley JV, Horie S. Localization of TRPV1 and contractile effect of capsaicin in mouse large intestine: high abundance and sensitivity in rectum and distal colon. Am J Physiol Gastrointest Liver Physiol 297: G348–G360, 2009. First published June 4, 2009; doi:10.1152/ajpgi.90578.2008.—We investigated immunohistochemical differences in the distribution of TRPV1 channels and the contractile effects of capsaicin on smooth muscle in the mouse rectum and distal, transverse, and proximal colon. In the immunohistochemical study, TRPV1 immunoreactivity was found in the mucosal, submucosal, and muscle layers and myenteric plexus. Large numbers of TRPV1-immunoreactive axons were observed in the rectum and distal colon. In contrast, TRPV1-positive axons were sparsely distributed in the transverse and proximal colon. The density of TRPV1-immunoreactive axons in the rectum and distal colon was much higher than those in the transverse and proximal colon. Axons double labeled with TRPV1 and protein gene product (PGP) 9.5 were detected in the myenteric plexus, but PGP 9.5-immunoreactive cell bodies did not colocalize with TRPV1. In motor function studies, capsaicin induced a fast transient contraction, followed by a large long-lasting contraction in the rectum and distal colon, whereas in the transverse and proximal colon only the transient contraction was observed. The capsaicin-induced transient contraction from the proximal colon to the rectum was moderately inhibited by an NK1 or NK2 receptor antagonist. The capsaicin-induced long-lasting contraction in the rectum and distal colon was markedly inhibited by an NK1 receptor antagonist, but not by an NK2 antagonist. The present results suggest that TRPV1 channels located on the rectum and distal colon play a major role in the motor function in the large intestine.

vanilloid; immunohistochemistry; afferent nerve; substance P; neuropeptide Y; neurokinin A

Capsaicin, the main pungent constituent in red peppers of the genusCapsicum, stimulates TRPV1 channels, leading to the activation of primary afferent neurons with unmyelinated or thinly myelinated nerve fibers, the so-called capsaicin-sensitive afferent neurons. Capsaicin has been utilized by numerous investigators to activate afferent fiber endings in studies of the visceral effects of TRPV1 in the gastrointestinal tract, and it has been established that capsaicin-sensitive primary afferent neurons participate in the regulation of gastrointestinal motility (8). For example, capsaicin induces contractions in the guinea pig ileum (8) and relaxation in human small and large intestines (7, 27, 28). Afferent nerves in the gut not only send signals to the central nervous system but also provide a local effenter-like effect by releasing neuropeptides. Capsaicin-sensitive nerve fibers that contain neuropeptides including tachykinins and calcitonin gene-related peptide have been identified in several mammalian species. In particular, tachykinins such as substance P and neurokinin A mediate the excitatory effect of capsaicin on sensory neurons (8). Neurochemical and functional evidence indicates that tachykinins are expressed in extrinsic and intrinsic primary afferent neurons (15, 21). The gastrointestinal tract contains two types of primary afferent neurons: intrinsic primary afferent neurons with cell bodies, processes, and synaptic connections in the gut wall, and extrinsic primary afferent neurons with their cell bodies in nodose and jugular ganglia or in dorsal root ganglia (16). TRPV1 channels are found predominantly on the sensory afferent nerve fibers implicated in pain transduction. TRPV1 nerve fibers in the gut appear to be predominantly extrinsic in origin (22, 31, 37), but their existence in intrinsic neurons in the gastrointestinal tract was also reported (3).

The TRPV1 channel has become an attractive target for treatment of colonic and rectal disorders such as irritable bowel syndrome and inflammatory bowel disease (2, 13, 39). However, most studies of the physiological roles of TRPV1 in motor function in the intestine have been carried out mainly using isolated guinea pig ileum. The effects of capsaicin and the distribution of TRPV1 channels in the lower gastrointestinal tract remain largely unstudied. In particular, regional and hence functional differences in TRPV1 among the rectum and distal, transverse, and proximal colon have never been reported. In the present study, we investigated the immunohistochemical differences in the distribution of TRPV1 channels and the contractile effect of capsaicin on smooth muscle in the mouse rectum and distal, transverse, and proximal colon. We also investigated the contractile mechanism of capsaicin in the proximal colon to the rectum and distal colon, whereas in the transverse and proximal colon only the transient contraction was observed.

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each segment of the lower gastrointestinal tract, with the special reference to the involvement of the tachykinin NK1 and NK2 receptors.

MATERIALS AND METHODS

Animals

Male ddY-strain mice (Japan SLC, Hamamatsu, Japan) 6–8 wk old were used. Animals were housed in a temperature-controlled room at 24°C with lights on from 0700 to 1900 and had free access to food and water. All experiments were performed in compliance with the “Guiding Principles for the Care and Use of Laboratory Animals” approved by the Japanese Pharmacological Society and the guidelines approved by the institutional Animal Care and Use Committee of Josai International University. The protocols used were approved by the committee of Josai International University in accordance with the guidelines of APS guiding principles in the care and use of animals. The number of animals used was kept to the minimum necessary for a meaningful interpretation of the data, and animal discomfort was kept to the minimum.

Drugs

The drugs used in this study were acetylcholine chloride, tetrodotoxin (TTX), capsaicin, and atropine sulfate (Wako Pure Chemical Industries, Tokyo, Japan), N-(4-tertiary butylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carbox-amide (BCTC) (Biomol International, Boechout, Belgium), N^2-nitro-l-arginine methyl ester (L-NAME), 1-(2-(5-fluoro-1H-indol-3-yl)ethyl)-4-methoxy-4-((phenylsulfonyl)methyl)piperidine (GR159897), substance P, neurokinin A (Sigma Chemical, St. Louis, MO), N^2-[4-(4'-hydroxy-1-(1-methyl-1H-indol-3-yl)carbonyl-1-l-propyl]-N-methyl-N-phenylmethyl-3-2-(2-naphthyl)l-alaninamide (FK888), and 3-methyl-2-phenyl-N-[1(S)-1-phenylpropyl]-4-quinoinecarboxamide (SB222200) (Tocris Cookson, Bristol, UK).

Capsaicin was dissolved in ethanol prior to dilution in deionized water. FK888, GR159897, SB222200, BCTC, and neurokinin A were dissolved in DMSO prior to dilution in deionized water. Other drugs were dissolved in deionized water with no organic solvent or detergents. The final concentrations of DMSO and ethanol in the bath were less than 0.2 and 0.1%, respectively. The vehicles used had no pharmacological effects on the tonus of preparations or the capsacin-induced contraction.

Isolated lower gastrointestinal tract and measurement of tension.

The preparation and measurement of contraction of segments of the mouse rectum and distal, transverse, and proximal colon were performed as described previously (32). Each segment was removed and placed in Krebs-Henseleit solution (in mM: 112.08 NaCl, 5.90 KCl, 1.97 CaCl_2, 1.18 MgCl_2, 1.22 NaH_2PO_4, 25.00 NaHCO_3, and 11.49 glucose). A segment of each tissue, ~1.0 cm length, was set up under a 0.7-g load in a 10-ml organ bath filled with Krebs-Henseleit solution. The optimal resting tension was determined by preliminary experiments. When the resting tension was 0.7 g, the maximum reproducible contraction by capsacin was obtained. The strips were mounted in the longitudinal direction, and longitudinal muscle tension was recorded. The bath was maintained at 37°C and continuously bubbled with a mixture of 95% O_2 and 5% CO_2. Contraction was recorded by use of an isotonic transducer (TD-112S, Nihon Kohden, Tokyo, Japan), a balancing box (JD-112S, Nihon Kohden), and a Powerlab system (AD Instruments, Castle Hill, Australia). At the start of each experiment, the responsiveness to acetylcholine at 10 μM, a submaximal concentration for acetylcholine-induced contraction, was ascertained to evaluate the contractile effects of the capsaicin, substance P, and neurokinin A. After at least three stable contractions were induced by 10 μM acetylcholine, the experiments were performed.

In the first series of experiments investigating the noncumulative concentration response to capsaicin, intestine segments were challenged only once with an individual dose of capsaicin (10 nM–10 μM) to avoid the influence of sensitization or desensitization on the contraction (10, 14). To investigate the possibility of desensitization to capsaicin in the contractile responses, the intestine preparations were incubated for 3 min with capsaicin (first treatment), then washed three times with the Krebs-Henseleit solution for 5 min each. After that, capsaicin at the same dose as the first treatment was added (second treatment). Each response was expressed as a percentage of the maximum contraction induced by 10 μM acetylcholine (% of acetylcholine contraction).

Mechanism of capsaicin-induced contraction. In studies of the effects of TTX, atropine, the nitric oxide synthase inhibitor L-NAME, or TRPV1 antagonist BCTC on capsaicin-induced contraction, each segment was preincubated with 300 nM of TTX for 10 min, 1 μM of atropine for 10 min, or 100 μM of L-NAME for 10 min, or 1 μM of BCTC for 45 min. In studies of the effects of the NK1 receptor antagonist FK888 and NK2 receptor antagonist GR159897, each segment was preincubated with 10 μM of FK888 or 3 μM of GR159897 for 20 min. The drug treatment protocols were previously described (4, 14). These drugs did not affect the baseline tension. Each response was expressed as a percentage of the maximum contraction induced by 10 μM acetylcholine (% of acetylcholine contraction).

Tissue preparation for immunohistochemistry. Segments of the mouse rectum and distal, transverse, and proximal colon were removed, fixed by immersion in fresh 4% paraformaldehyde in 0.1 M phosphate buffer for 2 h at 4°C, and washed three times with phosphate-buffered saline (PBS). They were cryoprotected overnight in 0.1 M phosphate buffer containing 20% sucrose. The tissues were frozen in OCT (Sakura Finetek, Toyo, Japan) mounting medium, and sectioned on a cryostat (Leica Instruments, Nussloch, Germany) at a thickness of 40 μm. The sections were thaw-mounted onto Superfrost Plus slides (Matsunami Glass, Osaka, Japan).

Immunohistochemical study. The immunohistochemical procedures were performed as described by Horie et al. (23) and Watanabe et al. (38). Prior to staining, the slide-mounted sections were successively incubated in 10% normal donkey serum containing 0.2% Triton X-100 and 0.1% sodium azide in PBS for 1 h, followed by PBS containing 0.3% hydrogen peroxide for 30 min to quench endogenous peroxidase activity, and were then washed three times for 10 min each with PBS. In addition, the avidin and biotin sites of sections were successively blocked using an avidin-biotin blocking kit (Vector Laboratories, Peterborough, UK), and the sections were then washed three times for 10 min each in PBS again. Subsequently, sections were incubated in rabbit polyclonal anti-TRPV1 antibody for 40 h at room temperature. Concentrations of the TRPV1 antibody (mouse TRPV1 C-terminus; Neuronics, Minneapolis, MN) were 1:60,000 for rectum, 1:40,000 for distal colon, and 1:20,000 for transverse and proximal colon (Figs. 1, 4, and 5). To investigate the difference of the density of TRPV1-immunoreactive nerve fibers in the rectum and distal, transverse, and proximal colon, two different anti-TRPV1 antibodies (1:60,000, Neuronics, and rat TRPV1 COOH-terminus, 1:1,000; Trans Genic, Kumamoto, Japan) were used as the primary antibody (Figs. 2 and 3). For immunoadsorption experiments, the antibody (1:60,000; Neuronics) was preincubated with 10 μM of the corresponding antigen peptide (Neuronics) fragment for 1 h at room temperature. After washes in PBS, the sections were incubated with biotinylated donkey anti-rabbit immunoglobulin G (1:400; Jackson Immunoresearch Laboratories, West Grove, PA) for 90 min at room temperature. After further washes, the sections were incubated in streptavidin biotin-peroxidase complex (1:5; Vectastain Elite ABC kit, Vector Laboratories) for 1 h at room temperature, followed by fluorescein isothiocyanate (FITC) tyramide (1:75; TSA kit, Perkin-Elmer Life Sciences, Boston, MA) (1). In control experiments, the TRPV1 antibody was omitted from the staining procedures to verify the specificity of the staining. No immunolabeling was observed in
these controls. Double staining of the sections with TRPV1 antiserum combined with a rabbit antiserum to the pan axonal marker protein gene product (PGP) 9.5 was also carried out. In the case of PGP 9.5, TRPV1 staining was carried out first by the TSA procedure described above with FITC as the label and then followed by PGP 9.5 staining (1:60,000; Biogenesis, Poole, UK) by the indirect labeling procedure. To visualize the PGP 9.5 labeling, sections were then incubated for 4 h with donkey anti-rabbit secondary antibody linked to tetramethyl rhodamine isothiocyanate (TRITC, 1:400; Jackson Immunoresearch Laboratories). There was no cross-reactivity between TRPV1 and PGP 9.5 staining.

Microscopy and image analysis. The sections were viewed at ×10 magnification (Zeiss Plan-NeoFluar) via an inverted fluorescence microscope (Axioskop2 plus, Zeiss, Göttingen, Germany) equipped with a filter for detection of fluorescein (FITC, 488 nm; TRITC, 543 nm). Randomly chosen images were acquired via a charge-coupled device camera (AxioCam MRm, Zeiss), stored in a personal computer, and analyzed with Zeiss imaging software (Axiovision LE version 4.6). To quantify TRPV1-positive axons, we measured TRPV1-immunopositive areas in the image using the Automeasure module (AutoMeasure Plux, Zeiss). Random fields were selected for each slide. Next, thresholds for the brightness that represents the TRPV1 immunoreactivity were selected by using the distribution of brightness information. The TRPV1-positive area was calculated by subtracting a nonspecific signal area of the control image (i.e., not exposed to the antibody) from that of the experimental image (i.e., antibody treated) on serial cryostat sections of rectum or distal, transverse, or proximal colon. In this manner, the absolute area of TRPV1-positive axons could be determined for individual experiments and calculated as follows: TRPV1-positive area (%) = [(TRPV1 positive area/total area of antibody-treated section) – (positive area of control section/total area of the control section)] × 100 (%).

For observation of the double staining of TRPV1 and PGP 9.5 in the submucosal layer and myenteric plexus, the sections were viewed at ×20 magnification (Olympus UPlanSApo) via a confocal microscope (FV-1000, Olympus, Tokyo, Japan). Multiple images in Z-stacks were projected onto a single plane.

Statistical analysis. The data are expressed as means ± SE. Statistical analyses were performed by the two-tailed Student’s *t*-test for comparison of two groups, and by a one-way analysis of variance followed by a Bonferroni multiple-comparison test for comparison of more than two groups. A *P* value < 0.05 was considered statistically significant.

RESULTS

Localization and quantification of TRPV1 immunoreactivity in isolated mouse lower gastrointestinal tract. Numerous TRPV1-immunoreactive nerve fibers were seen in the mucosa, submucosal layer, and myenteric plexus of the rectum and distal colon (Fig. 1, A, B, and D). TRPV1-immunoreactive fibers were also observed in the muscle layer and around bundles of arterioles, venules, and lymphatic vessels in the submucosal layer (Fig. 1C). TRPV1-immunoreactive fibers were not observed after preincubation of the antibody with the antigen peptide (Fig. 1E).
The difference in the distributions of TRPV1 in mouse large intestine was examined by using serial cryostat sections of the rectum and distal, transverse, and proximal colon under the same immunostaining condition (Fig. 2). To measure the amount of TRPV1 immunoreactivity, two different anti-TRPV1 antibodies (Neuromics and Trans Genic) were used as the primary antibody. In the rectum, numerous TRPV1-immunoreactive nerve fibers were found in the mucosa, submucosal layer, and muscle layer and myenteric plexus compared with control tissues (Fig. 2, A and B). In the distal colon, the density of TRPV1-positive nerve fibers was lower than in the rectum, but they were clearly observed in the submucosal layer and myenteric plexus compared with control tissues (Fig. 2, D and E). In contrast, the TRPV1-positive axons were sparsely distributed in the transverse and proximal colon (Fig. 2, G, H, J, and K). Thus similar results were obtained from both experiments using two different anti-TRPV1 antibodies. In control experiments without the corresponding primary antibody, no immunolabeling was observed (Fig. 2, C, F, I, and L).

To better understand the difference between the amounts of TRPV1-immunoreactive nerve fibers in the rectum and distal, transverse, and proximal colon, TRPV1-immunoreactive areas were quantified by computerized binary image analysis (Fig. 3). Figure 3 shows TRPV1-immunoreactive areas in all layers of the transverse section (Fig. 3A) and in the muscle layer including myenteric plexus (Fig. 3B) using a TRPV1 antibody (Neuromics). The TRPV1-immunopositive area in the rectum was the largest in the isolated mouse lower gastrointestinal tract. Both the rectum and distal colon contained a significantly higher density of TRPV1-immunoreactive nerve fibers than the transverse and proximal colon. The density of TRPV1-positive nerve fibers was very low in the transverse and proximal colon. These results were further confirmed by an immunohistochemical experiment using another TRPV1 antibody (Trans Genic, Fig. 3, C and D).

Colocalization of TRPV1 with PGP 9.5. To investigate the precise localization of TRPV1, we performed double-labeling experiments with PGP 9.5, a pan axonal marker, to identify neuronal cell bodies and nerve fibers using the fluorescence microscope (Fig. 4) and confocal microscope (Fig. 5). Abundant TRPV1 immunoreactivity was detected in the myenteric and submucosal plexuses of each part of the lower gastrointestinal tract (Fig. 4, A, D, G, and J). In all cases, the immunoreactivity appeared axonlike in nature. TRPV1 staining of ganglionic cells was not detected in either submucosal or myenteric plexuses in the rectum. PGP 9.5 staining was widely distributed within the mucosa, submucosa, and smooth muscle layer of each part of the lower gastrointestinal tract (Fig. 4, B, E, H, and K) and did not show a clear difference among the parts. Abundant PGP 9.5-immunoreactive fibers and bundles were found in the myenteric plexus and submucosal layer, and TRPV1 immunoreactivity profiles in these areas showed dou-
ble labeling with PGP 9.5 (Fig. 4, C, F, I, L). Figure 5 shows the high-power magnification confocal images of the submucosal layer and myenteric plexus of rectum. Many TRPV1/PGP 9.5 double-labeled axons were observed, but PGP 9.5-immunoreactive cell bodies did not colocalize with TRPV1.

**Difference in capsaicin-induced contractions in isolated mouse rectum, distal, transverse, and proximal colon.** Next, we investigated the effect of the contraction induced by the TRPV1 agonist capsaicin on smooth muscle tension in the isolated mouse lower gastrointestinal tract. Figure 6 shows typical recordings and concentration-response curves of the capsaicin-induced contraction in mouse rectum and distal, transverse, and proximal colon. In the rectum and distal colon, capsaicin induced fast transient contractions, followed by slowly developing long-lasting contractions that peaked within 2–3 min after administration (Fig. 6, A and B). The contractions induced by capsaicin were concentration dependent. However, only transient contractions were observed in the transverse and proximal colon (Fig. 6, C and D). No statistical differences were found between 1 μM capsaicin-induced transient contractions in each part of the lower gastrointestinal tract. Preeposure of tissues to capsaicin caused a dose-dependent desensitization to the second application of capsaicin in all parts of the isolated lower gastrointestinal tract.

**Effects of TTX, atropine, and TRPV1 antagonist BCTC on capsaicin-induced contraction in isolated mouse lower gastrointestinal tract.** To clarify the mechanism underlying contractile responses to TRPV1 activation by capsaicin in the mouse rectum and distal, transverse, and proximal colon, we evaluated the effects of the neurotransmission blocker TTX, muscarinic receptor antagonist atropine, and TRPV1 antagonist BCTC on those contractile responses (Table 1). TTX abolished transient contractile responses to capsaicin from the rectum to the proximal colon, compared with each control group. Capsaicin-induced long-lasting contractions were significantly but partially inhibited by TTX in the rectum and distal colon. Atropine abolished the transient contraction from the rectum to the proximal colon. The long-lasting contractions were significantly but partially inhibited by atropine in the rectum and distal colon. The TRPV1 antagonist BCTC almost completely inhibited both transient and long-lasting contractions in all parts of the isolated mouse lower gastrointestinal tract. BCTC (1 μM) did not affect the direct smooth muscle contractions in response to 10 μM of acetylcholine (data not shown), but the long-lasting contraction was not abolished by BCTC. Then we performed a preliminary experiment with another TRPV1 antagonist, iodoresiniferatoxin, but it also failed to abolish the capsaicin-induced long-lasting contraction (data not shown).

To investigate the capsaicin-induced relaxation in the mouse colon, inhibition by nitric oxide synthase was examined. The distal colon segments were preincubated with the nitric oxide synthase inhibitor L-NAME (100 μM). L-NAME did not affect the capsaicin-induced transient contractions in the distal colon (control 33.1 ± 3.4, L-NAME 31.34 ± 12.6; n = 3–6) or long-lasting contractions in the distal colon (control 38.0 ± 2.7, L-NAME 33.6 ± 4.2; n = 3–6).

**Effects of tachykinin-receptor antagonists on capsaicin-induced contraction in isolated mouse lower gastrointestinal tract.** Tachykinins play an important role in the contractile effect of capsaicin: they stimulate myenteric neurons and mediate the neurogenic excitatory effects of capsaicin. We
investigated the contractile effects of the tachykinins substance P and neurokinin A on capsaicin-induced contraction in the isolated mouse lower gastrointestinal tract (Fig. 7). Preliminary experiments revealed that substance P (10 nM–1 μM) and neurokinin A (10 nM–3 μM) induced dose-dependent contractions in the isolated mouse lower gastrointestinal tract. Therefore, we investigated the contractile response to substance P and neurokinin A in the mouse rectum and distal, transverse, and proximal colon (Fig. 7, A–D). The concentrations of substance P and neurokinin A were chosen to induce submaximal contractions in each part. Substance P produced fast transient contractions in all parts of the mouse isolated lower gastrointestinal tract. Neurokinin A produced large long-lasting contractions in the rectum and distal colon that peaked 2–3 min after administration. In the transverse and proximal colon, neurokinin A induced fast transient contractions, followed by small long-lasting contractions. To determine the contribution of the cholinergic pathways, responses to substance P and neurokinin A were compared in the presence and absence of the muscarinic receptor antagonist atropine (Fig. 7, A–D). Atropine partially and significantly inhibited the substance P-induced contractions, but no significant effect on the neurokinin A-induced contractions was observed in any part of the mouse lower gastrointestinal tract.

Fig. 4. Colocalization of TRPV1-positive nerve fibers with protein gene product (PGP) 9.5 in mouse rectum and distal, transverse, and proximal colon. Mouse lower gastrointestinal sections were double labeled with TRPV1 (green) and PGP 9.5 (red). The TRPV1 and PGP 9.5 labelings are shown separately (A, D, G, J) and (B, E, H, K), respectively, and merged (C, F, I, L). TRPV1 axons are sparse in the smooth muscle layer but prominent in the myenteric nerve plexus (arrows) and submucosal layer (concave arrowheads). In contrast, PGP 9.5 immunoreactive axons are abundant in all regions. Arrows and concave arrowheads indicate the colocalization of TRPV1 immunoreactivity with PGP 9.5 immunoreactivity. Scale bars are 100 μm.

Fig. 5. Confocal images showing TRPV1 and protein gene product (PGP) 9.5 double labeling in the submucosal layer (A–C) and myenteric plexus (D–F) of the mouse rectum. Sections were double labeled with TRPV1 (green) and PGP 9.5 (red). The TRPV1 and PGP 9.5 labelings are shown separately in (A and D) and (B and E), respectively, and merged (C and F). Arrowheads and arrows indicate the colocalization of TRPV1/PGP 9.5 double-labeled axons in the submucosal layer and myenteric plexus, respectively. Scale bar is 50 μm.
Next, we evaluated the effects of the NK₁ receptor antagonist FK888 and the NK₂ receptor antagonist GR159897 on capsaicin-induced contractile responses (Fig. 8). Capsaicin-induced transient contractions from the rectum to the proximal colon were significantly and moderately inhibited by FK888 or GR159897 compared with control responses (Fig. 8, A–D). The combined blockade of NK₁ and NK₂ receptors with FK888 and GR159897 markedly inhibited transient contractile responses to capsaicin from the rectum to the proximal colon. The long-lasting contractions in the rectum and distal colon were significantly and moderately inhibited by GR159897, but not by FK888 (Fig. 8, A and B). Coadministration of FK888 and GR159897 markedly inhibited long-lasting contractile responses to capsaicin in the rectum and distal colon.

The substance P (100 nM)-induced contractions in the rectum and distal, transverse, and proximal colon were significantly blocked by 10 μM acetylcholine (% of ACh response) in each part of the lower gastrointestinal tract. Each value represents means ± SE of data obtained from 3–7 mice. No statistical differences were found on 1 μM capsaicin-induced transient contraction among each part of lower gastrointestinal tract.

Fig. 6. Typical recordings and concentration-response curves of capsaicin-induced contraction and desensitization in mouse rectum (A) and distal (B), transverse (C), and proximal colon (D). Typical recordings show that capsaicin induced a transient contraction, followed by a long-lasting contraction in the rectum and distal colon. In the transverse and proximal colon, only the transient contraction was observed. Data for each contraction are expressed as a percentage of the maximal contraction induced by 10 μM acetylcholine (% of ACh response) in each part of the lower gastrointestinal tract. Each value represents means ± SE of data obtained from 3–7 mice. No statistical differences were found on 1 μM capsaicin-induced transient contraction among each part of lower gastrointestinal tract.
Table 1. Effect of TTX, atropine, or TRPV1 antagonist BCTC on capsaicin-induced contraction in isolated mouse rectum, distal colon, transverse colon, and proximal colon

<table>
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<tr>
<th>Segment</th>
<th>Control</th>
<th>TTX, 300 nM</th>
<th>Atropine, 1 M</th>
<th>BCTC, 1 M</th>
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<tr>
<td>Rectum</td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>Transient</td>
<td>33.2±5.5</td>
<td>1.6±1.6†</td>
<td>1.3±1.3†</td>
<td>3.1±0.6†</td>
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<tr>
<td>Long-lasting</td>
<td>50.4±3.4</td>
<td>36.7±3.5*</td>
<td>35.8±5.4*</td>
<td>7.0±0.3†</td>
</tr>
<tr>
<td>Distal colon</td>
<td>33.1±3.4</td>
<td>0.0±0.0†</td>
<td>0.5±0.3†</td>
<td>3.7±1.2†</td>
</tr>
<tr>
<td>Transient</td>
<td>38.6±2.7</td>
<td>25.4±3.3*</td>
<td>14.3±4.6†</td>
<td>5.7±1.9†</td>
</tr>
<tr>
<td>Proximal colon</td>
<td>22.3±3.7</td>
<td>0.0±0.0†</td>
<td>3.7±2.2†</td>
<td>1.6±0.3†</td>
</tr>
<tr>
<td>Transient</td>
<td>30.0±5.5</td>
<td>0.0±0.0†</td>
<td>6.4±2.4*</td>
<td>1.5±0.3†</td>
</tr>
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</table>

Each value represents the mean ± SE of 5–7 animals. TTX, tetrodotoxin; BCTC, N-(4-tertiary butylphenyl)-4-(3-chloropyridin-2-yl)tetrahydropyrazine-1(2H)-carboxamide. Values that were significantly different from those of the control group: *P < 0.05; †P < 0.01.

60.8 ± 8.2, FK888 18.4 ± 3.8%, P < 0.01; n = 5–8), but not significantly by 3 μM GR159897 (rectum: control 59.2 ± 7.9, GR159897 49.9 ± 8.5; distal colon: control 66.6 ± 11.6, GR159897 61.9 ± 12.6; transverse colon: control 61.3 ± 11.3, GR159897 53.8 ± 10.4; proximal colon: control 60.8 ± 8.2, GR159897 62.4 ± 10.8%; n = 5–8). The neurokinin A (300 nM or 1 μM)-induced contractions in the rectum and distal, transverse, and proximal colon were significantly blocked by 3 μM GR159897 (rectum: control 72.7 ± 15.9, GR159897, 9.4 ± 2.6, P < 0.05; distal colon: control 65.3 ± 16.5, GR159897 18.4 ± 4.5, P < 0.05; transverse colon: control 46.0 ± 8.5, GR159897 9.0 ± 2.7, P < 0.01; proximal colon: control 31.2 ± 4.1, GR159897 6.8 ± 1.6%, P < 0.01; n = 5–8), but not significantly by 10 μM FK888 (rectum: control 72.7 ± 15.9, FK888, 81.1 ± 13.8; distal colon: control 65.3 ± 16.5, FK888 79.3 ± 19.3; transverse colon: control 46.0 ± 8.5, FK888 47.7 ± 18.7; proximal colon: control 31.2 ± 4.1, FK888 36.4 ± 8.4%; n = 5–8). An NK3 receptor blockade partially inhibited the contraction induced by capsaicin, suggesting the involvement of NK3 receptors in the response to capsaicin in mouse jejunal (14). We tried to clarify this by using the commercially available NK3 antagonist SB222200 (30). SB222200 (30 μM) inhibited the neurokinin B-induced contraction, but it also markedly inhibited the substance P- and neurokinin A-induced contractions. Therefore, the involvement of NK3 receptors in capsaicin-induced contraction is unclear at this time (data not shown).

DISCUSSION

This study was designed to investigate differences in the distribution of TRPV1 channels and the effects of capsaicin on smooth muscle contraction using isolated mouse lower gastrointestinal tract segments. The localization of TRPV1 channels and their effects on motor function in the large intestine, especially site differences, remain to be elucidated. In the present study, we isolated the mouse rectum and distal, transverse, and proximal colon and examined the pharmacological and physiological differences among the parts by using immunohistochemical methods and tension recordings of capsaicin-induced contractions. The present results suggest that TRPV1 channels located on the rectum and distal colon play a major role in the motor function of the mouse lower gastrointestinal tract. Furthermore, neurokinin A was found to be the major neurotransmitter in large long-lasting capsaicin-induced contractions in the mouse rectum and distal colon.

First, we mapped the localization and distribution of TRPV1 in the mouse lower gastrointestinal tract using immunofluorescence. To increase the sensitivity of immunodetection, we visualized anti-TRPV1 antibodies using an amplification method based on the catalyzed deposition of fluorescein-conjugated tyramide (1). In this study, we demonstrate TRPV1 immunoreactivity in the mucosa, submucosal layer, muscle layer, and myenteric plexus of mouse lower gastrointestinal tract. In the mucosal layer of the rectum and distal colon, TRPV1-immunoreactive axons were observed running across and along the mucous glands. In the submucosal layer, TRPV1-immunoreactive fibers were found around bundles of arterioles, venules, and lymphatic vessels. In our previous study of rat stomach, TRPV1 axons were observed running along gastric glands in the mucosa and around blood vessels in the submucosal layer, suggesting the role of TRPV1 nerve fibers in gastric acid and mucus secretion (22, 23). The gastrointestinal protection produced by capsaicin-induced stimulation of sensory neurons is associated with a marked increase of mucosal blood flow in the small and large intestine of experimental animals (18). These findings suggest that the TRPV1 nerve fibers of the mucosal layer are involved in mucus secretion and blood flow in the mouse large intestine. In the muscle layer, TRPV1-immunoreactive fibers were observed in the rectal side of the mouse lower gastrointestinal tract.

Extrinsic primary afferent neurons have their cell bodies in nodose and jugular ganglia or dorsal root ganglia. TRPV1-positive nerve fibers in the gut appear to originate predominantly in spinal afferents, with very few in vagal afferents (37). Other investigators reported that TRPV1 immunoreactivity was detected on both intrinsic and extrinsic neurons in the gut (3), and TRPV1 mRNA has also been reported in the mouse colon (25, 32). Ward et al. (37) reported that TRPV1 nerve fibers form a network around myenteric nerve cell bodies in the mouse, guinea pig, and rat gastric antrum. Consistent with previous findings for TRPV1/PGP 9.5 double staining (23, 37) in the gastrointestinal tract, we observed TRPV1/PGP 9.5 double-labeled axons in the myenteric plexus. However, PGP 9.5-immunoreactive cell bodies were not colocalized with TRPV1 immunoreactivity. Pharmacological studies have shown that the population of capsaicin-sensitive gastrointestinal neurons is composed of extrinsic primary afferents, whereas intrinsic enteric neurons do not respond directly to capsaicin (8). Taken together, the data imply that TRPV1-expressing nerve fibers observed in the myenteric plexus are extrinsic primary afferents, but further studies are needed to determine the origin of the TRPV1 channels in the mouse large intestine.

The difference between the densities of TRPV1-immunoreactive nerve fibers in the rectum and distal, transverse, and proximal colon has not been studied under physiological conditions. In the present study, the TRPV1-immunoreactive areas of each segment were quantified by computerized binary image analysis using two anti-TRPV1 antibodies. Interestingly, high densities of TRPV1-positive areas were detected in the rectum and distal colon, but not in the transverse and proximal colon. In contrast, clear differences in PGP 9.5 densities were not
observed from the proximal colon to the rectum. Consistent with the binary image analysis of the muscle layers including the myenteric plexus in each segment, the capsaicin-induced contraction patterns were different on the anal (rectum and distal colon) and oral (transverse and proximal colon) sides of the mouse large intestine. These contraction patterns were characterized by transient and subsequent long-lasting contractions in the rectum and distal colon and only transient contractions in the transverse and proximal colon. Thus we speculated that TRPV1 channels located on the rectum and distal colon play a major role in the motor function. On the basis of our in vitro data, we speculated that capsaicin stimulates colonic motility in the in vivo experiment. Indeed, it is reported that intracolonic administration of capsaicin increases colonic motility in a dose-dependent manner in conscious dogs (17). Therefore, the activation of TRPV1 channels is thought to result in the promotion of motility in the colon.

TRPV1 channels located in the mucosa are considered to play important roles in detecting visceral pain because TRPV1 contributes to the perception of noxious mechanical stimuli and inflammatory stimuli in the mouse colon (24, 29). In the gastrointestinal tract, TRPV1 channels play both protective and nonprotective roles against inflammatory colitis (35). Holzer (20) suggested that TRPV1 channels involved in gastrointestinal protection may be differentiated pharmacologically from TRPV1 channels mediating gastrointestinal inflammation. Azuma et al. (6) reported that the histological colitis score of the distal colon was higher than that of the proximal colon in dextran sodium sulfate-induced colitis. In this study, we clearly demonstrated that the TRPV1 channel density in the mucosal area is greater in the rectum and distal colon than in the transverse and proximal colon. Thus the abundant TRPV1 channel expression may result in the induction of more pronounced colitis in the rectum and distal colon than in the transverse and proximal colon. However, further studies are needed to clarify the roles of TRPV1 channels in colitis.

In the guinea pig esophagus, capsaicin induces a biphasic contraction, i.e., immediate transient and late long-lasting contractions (9). A similar contraction pattern was observed in the mouse rectum and distal colon in the present study. The contraction due to capsaicin is well studied in guinea pig ileum, and it is known that biologically active substances released from sensory nerves stimulate both smooth muscle and intrinsic myenteric neurons (8). Acetylcholine released from intrinsic cholinergic motor neurons is one of the final mediators of the capsaicin-induced contraction. Capsaicin-sensitive sensory nerves innervating the gastrointestinal tract are known to be TTX resistant (5, 11). In the guinea pig ileum, capsaicin induces TTX-sensitive acetylcholine release, suggesting that the TTX- and atropine-sensitive contractile effects of capsaicin are based on acetylcholine release from myenteric cholinergic neurons stimulated by neurotransmitters from sensory nerves (34). On the other hand, the TTX-insensitive contractile effect of capsaicin is attributed to a direct activation of smooth muscle stimulated by neurotransmitters from sensory nerve endings (14). In the present study, capsaicin-induced transient contractions in the rectum to proximal colon were abolished by TTX and atropine. The long-lasting contractions in the rectum and distal colon were partially inhibited by TTX and atropine. The amplitudes of transient and long-lasting contractions were reduced by repeated administration of capsaicin, which is one peculiar effect of TRPV1 activation. To study the involvement of TRPV1 in isolated gastrointestinal preparations, we used BCTC as a selective TRPV1 antagonist (33). BCTC almost completely inhibited the response to capsaicin in all gastrointestinal segments without affecting the direct smooth muscle contraction, as previously reported for isolated mouse jejunum (14). However, we observed that the long-lasting contraction was not abolished even with adequate BCTC treatment. Next, we performed a preliminary experiment with another TRPV1 antagonist, iodoresiniferatoxin, but iodoresiniferatoxin also failed to abolish the capsaicin-induced long-lasting contraction. Therefore, capsaicin is thought to elicit the long-lasting contraction partly via the TRPV1-independent pathway. De Man et al. (14) also reported that TRPV1 antagonist iodoresiniferatoxin partially inhibited the contraction to capsaicin in mouse jejunum.

Benko et al. (10) reported that capsaicin induces nitric oxide-mediated relaxation in circular muscle-oriented preparations isolated from human and mouse colon. In the present study, we measured the longitudinal tension in longitudinally oriented muscle preparations. Pretreatment with l-NAME did not affect the capsaicin-induced contraction in the mouse distal colon, suggesting that nitric oxide is not involved in this response. Furthermore, in our preliminary experiments, we never observed nitric oxide-mediated relaxation in colonic and rectal longitudinal muscle treated with capsaicin when the isolated segments were preincubated with the NK1 antagonist FK888, the NK2 antagonist GR159897, or atropine, unlike the report of Benko et al. It is thought that the difference between Benko’s and our results arises from the preparations used.

Taken together, these results suggest that the capsaicin-induced transient contraction is mediated by intrinsic cholinergic activation, followed by the release of sensory neurotransmitters from the extrinsic sensory nerve. The long-lasting contraction is mediated mainly by neurotransmitters released from extrinsic sensory nerve endings and partially by the cholinergic pathway in the mouse lower gastrointestinal tract.

It is well known that tachykinins released from capsaicin-sensitive primary afferents elicit contractions of smooth muscle by a local efferent action (8). The involvement of tachykinin NK1 and NK2 receptors, which prefer substance P and neurokinin A, respectively, in the modulation of capsaicin-induced contractions has been shown in the guinea pig ileum. The distributions of substance P, neurokinin A, NK1 receptors, and NK2 receptors have been studied by immunohistochemical

Fig. 7. Typical recordings showing contractile response to substance P and neurokinin A in mouse rectum (A) and distal (B), transverse (C), and proximal colon (D). The concentrations of substance P and neurokinin A used in each segment were that same as shown in each typical recording. Data are expressed as a percentage of the maximal contraction induced by 10 μM acetylcholine in each part of the lower gastrointestinal tract. Each value represents means ± SE of data obtained from 5–8 mice. *Significantly different from those of the control by Student’s t-test (*P < 0.05, **P < 0.01).
methods. Substance P and neurokinin A are expressed in extrinsic and intrinsic primary afferent neurons and myenteric neurons (interneurons, excitatory motor neurons) (26). NK1 receptor immunoreactivity has been found in the myenteric plexus and muscle layer (26, 36). The NK2 receptors are found in longitudinal and circular muscle fibers of the mouse ileum and are thought to be expressed mainly on the smooth muscle layer (26, 31). To investigate the pattern of contraction induced by the NK1 and NK2 receptor activation, we compared exogenous substance P- and neurokinin A-induced contractions, respectively. We observed atropine-sensitive, fast transient contractions induced by substance P in all parts of the isolated mouse lower gastrointestinal tract. Neurokinin A produced large atropine-insensitive, long-lasting contractions in the rectum and distal colon. In the transverse and proximal colon, neurokinin A induced fast transient contractions, followed by small long-lasting contractions. The reactivities to neurokinin A of the rectum and distal colon were higher than those of the proximal colon. The reactivities to neurokinin A of the rectum and distal colon were higher than those of the proximal colon.
transverse and proximal colon. It is suggested that NK1 and NK2 receptor activation induces a capsaicin-like contractile response in the mouse lower gastrointestinal tract.

Therefore, we next investigated the effects of the NK1 receptor and/or the NK2 receptor blockade on capsaicin-induced contractile responses. Our results show that the capsaicin-induced transient contraction was significantly inhibited by NK1 or NK2 receptor antagonists, and a combined blockade of NK1 and NK2 receptors abolished transient contractile responses to capsaicin, suggesting that NK1 and NK2 receptors cooperate in producing the transient contraction. On the other hand, the long-lasting capsaicin-induced contraction was significantly inhibited by an NK2 antagonist but not by an NK1 antagonist, indicating that the release of neurokinin A from sensory nerve endings is mainly responsible for the long-lasting contraction. Altogether, these results indicate that different mechanisms are involved in the transient and long-lasting contractions induced by capsaicin in the mouse lower gastrointestinal tract, i.e., the intrinsic cholinergic and extrinsic sensory pathways are the main pathways of transient and long-lasting contractions, respectively. Substance P and neurokinin A released from sensory nerves stimulate NK1 and NK2 receptors on parasympathetic ganglia, leading to acetylcholine release from cholinergic nerve endings. This is the putative mechanism of capsaicin-induced transient contractions in the rectum and proximal colon. In the long-lasting contraction in the rectum and distal colon, the major final mediator is the neurokinin A released from extrinsic sensory nerve endings, which activates NK2 receptors on the smooth muscle cells. Our results suggest that TRPV1 channels located on the rectal and distal colon play a major role in the motor function of the large intestine. The differences between capsaicin-induced contractions in the rectum, distal colon, transverse colon, and proximal colon segments may be attributed to these differences in the TRPV1 density and neurokinin A reactivity. Although the present study provides significant information on the mechanisms of capsaicin-induced contractions in the mouse lower gastrointestinal tract, the localization of endogenous tachykinins and tachykinin receptors remains unknown. Colocalization studies on tachykinins and/or tachykinin receptors with TRPV1 channels may support the involvement of tachykinins, especially neurokinin A and NK2 receptors, in the mechanism of TRPV1-mediated motor function of the rectum and distal colon.

The present results provide new information regarding the site specificity of TRPV1 channels in the mouse lower gastrointestinal tract. Our findings suggest that TRPV1 channels located on the rectum and distal colon play a major role in the motor function of the large intestine. Further studies of TRPV1 channels in the rectum and distal colon may provide significant information about the role of TRPV1 in regulating a wide variety of functions in the large intestine.

ACKNOWLEDGMENTS

We thank Dr. Naoto Watanabe (Department of Respiratory and Infectious Diseases, St. Marianna University School of Medicine; Laboratory of Pharmacology, Faculty of Pharmaceutical Sciences, Josai International University) for helpful advice and for discussions of the immunohistochemical study.

GRANTS

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture of Japan (nos. 18590156, 18790126, 18790127, and 20790075) and by the Uehara Memorial Foundation.

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