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Full paper

Contraction of gut smooth muscle cells assessed by fluorescence imaging

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ABSTRACT

Here we discuss the development of a novel cell imaging system for the evaluation of smooth muscle cell (SMC) contraction. SMCs were isolated from the circular and longitudinal muscular layers of mouse small intestine by enzymatic digestion. SMCs were stimulated by test agents, thereafter fixed in acrolein. Actin in fixed SMCs was stained with phalloidin and cell length was determined by measuring diameter at the large end of phalloidin-stained strings within the cells. The contractile response was taken as the decrease in the average length of a population of stimulated-SMCs. Various mediators and chemically identified compounds of daikenchuto (DKT), pharmaceutical-grade traditional Japanese prokinetics, were examined. Verification of the integrity of SMC morphology by phalloidin and DAPI staining and semi-automatic measurement of cell length using an imaging analyzer was a reliable method by which to quantify the contractile response. Serotonin, substance P, prostaglandin E_2 and histamine induced SMC contraction in concentration-dependent manner. Two components of DKT, hydroxy-α-sanshool and hydroxy-β-sanshool, induced contraction of SMCs. We established a novel cell imaging technique to evaluate SMC contractility. This method may facilitate investigation into SMC activity and its role in gastrointestinal motility, and may assist in the discovery of new prokinetic agents.

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1. Introduction

Through changes in their contraction-relaxation cycle, smooth muscle cells (SMCs) play an essential role in the maintenance of biological homeostasis and SMC contractility is a key component of gastrointestinal motility studies. Until now, gut smooth muscle mechanism has been investigated by measuring the contraction-relaxation of isolated muscle strips (MS). However, the muscular layer of the gastrointestinal tract is complex, containing the enteric nerve plexus with intrinsic and extrinsic neurons, interstitial cells of Cajal (ICC), enteric glia, and macrophages, as well as longitudinal and circular SMCs (1). Therefore, MS responses are a composite of the actions from a variety cell types and specific detail regarding SMC activity difficult to be obtained. In order to further investigate the biology of the gastrointestinal motility, both techniques using MS and SMC are necessary to fully elucidate the mechanism of contractile response. In analysis of SMC level, SMCs are routinely obtained from muscle tissue by enzymatic digestion, after which the contractile response can be calculated by measuring cell length by fixation or perfusion methods (2,3). However, these procedures generally require a great amount of time and technical skill. Neither method can avoid arbitrarily selecting “usable” SMC given that selection is solely dependent on morphological observation by phase-contrast light microscopy.

Several neurotransmitters and inflammatory mediators induce contraction-relaxation of smooth muscle, which is a complex multidirectional interaction between immune and inflammatory

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cells, neurons and SMCs (4). Effects on SMC can be distinguished from neurally mediated effects by measuring membrane depolarization using isolated MS or by measuring isolated MS contractile responses in the presence of tetrodotoxin, which blocks Na^+ action potentials in most neurons, as well as transmission along nerves and spontaneous and evoked Ca^{2+} transients in myenteric neurons (5,6). Thus, the contraction activity of various transmitters at SMC level, especially gut SMCs, has been still insufficiently documented.

In the present study, we first developed and optimized an automated cell imaging protocol by which to quantify the contractility of SMCs. This protocol is based on the conventional and well-used method of a contractility assay; however, the introduction of fluorescent dyes as well as an algorithm for automatic measurement of cell length enables a more convenient and reliable estimation of SMC contractility. Secondly, we aimed to assess contractility in response to various neurotransmitters and inflammatory mediators which are thought to induce SMC contractility. Furthermore, we examined several components of daikenchuto (DKT), a pharmaceutical-grade traditional Japanese (Kampo) medicine, which is widely prescribed to relieve postoperative ileus in Japan (7). DKT is currently undergoing a number of double-blind placebo-controlled clinical trials for various intestinal diseases in the USA, one of which clearly demonstrates that DKT accelerates gastrointestinal transit in healthy humans (8). Pharmacokinetic studies have now established the pharmacologically active components of DKT by looking at plasma concentration profiles (9,10). Since DKT contains a number of agents capable of stimulating a contractile response of MS, we examined whether these components might have an effect on SMC contractility using our new method.

2. Materials and methods

2.1. Reagents

The following materials were used in this study: carbamylcholine chloride (CCh), serotonin hydrochloride (5-HT), substance P acetate salt hydrate (SP), prostaglandin E_2 (PGE_2), histamine dihydrochloride, and capsaicin. Collagenase (*Clostridium histolyticum* type 1), trypsin inhibitor (typeI-s: from soybean), and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Acrolein monomer was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). 4′ 6-diamino-2-phenylindole dihydrochloride (DAPI) and Alexa Fluor 488 phalloidin were obtained from Invitrogen (Eugene, OR). Hydroxy-α-sanshool (HAS) and hydroxy β-sanshool (HBS) were extracted from zanthoxylum fruits at Tsumura & Co. (Tokyo, Japan). Potassium chloride (KCl), [6]-shogaol, [6]-gingerol, ginsenoside Rb1 and ginsenoside Rg1 were purchased from Wako Pure Chemicals Industries (Osaka, Japan). Other reagents used for analysis were purchased from commercial sources.

2.2. Mice

Seven-week-old male BALB/c mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Studies were performed on male BALB/c mice aged 8–10 weeks. The animals were allowed free access to water and standard laboratory food (MF, Oriental Yeast, Tokyo, Japan) and housed in an animal room kept at a temperature of 23 ± 1 °C, a relative humidity of 55 ± 5%, and controlled lighting with the lights on from 7:00 to 19:00 daily. All experimental procedures were performed according to the 'Guidelines for the Care and Use of Laboratory Animals' approved by the Laboratory Animal Committee of Tsumura & Co. (approved protocol No. 09–24, 09–44, 10–016).

2.3. Immunohistochemistry

Intestinal specimens were opened along the mesenteric border. The specimens were stretched taut and pinned out flat to a silicone ring and fixed with ice cold acetone for 30 min. After fixation, the preparations were washed three times for 10 min each in phosphate-buffered saline (0.9% NaCl in 0.1 M sodium phosphate buffer, pH 7.0). The preparations were placed in Superblock (Thermoscientific, Rockford, IL) containing 0.3% Triton X-100 overnight at 4 °C. The preparations were then placed in primary antibody diluted in antibody diluent (DAKO Japan, Tokyo Japan) overnight at 4 °C. After removal from the primary antibody, the tissues were rinsed for 3 × 10 min with PBS and incubated with the relevant secondary antibodies conjugated to Alexa fluorochromes (Molecular Probes, Eugene, OR) diluted in antibody diluent (DAKO) overnight at 4 °C. After a final set of rinses, the preparations were mounted on microslides and coverslipped with Prolong Gold antifade reagent (Molecular Probes). The slides were observed using confocal laser microscopy FV-100D (Olympus, Tokyo, Japan). The following antibodies were used: a guinea pig polyclonal antibody to PGP9.5 (Abcam), a mouse monoclonal antibody to GFAP (GA5, Cell Signaling Technologies, Beverly, MA), a mouse monoclonal antibody to neurofilament 200 (clone NE14, Sigma), a rat monoclonal antibody F4/80 (A3-1, AbD), and a polyclonal rabbit anti-human CD117 antibody (DAKO). Smooth muscle actin was visualized by staining with Alexa568-conjugated phalloidin (Molecular Probes).

2.4. Preparation of dispersed SMCs

SMCs were isolated from the muscular layer of BALB/c mouse intestine using a previously described method (11). Mice were sacrificed by cervical dislocation. The small intestine was removed and the muscular layer was carefully peeled away. The peeled muscular layer included the longitudinal and circular smooth muscle layers. The muscular layer was incubated for two successive 10-min periods at 31 °C in HEPES medium containing (in mM) 98.1 NaCl, 3.87 KCl, 2.42 $NaH_2PO_4H_2O$, 4.86 L-glutamic-acid, 4.86 fumaric acid, 4.86 pyruvate, 11.17 glucose, 1.79 $CaCl_2$, 1.2 $MgSO_47H_2O$ and 23.5 HEPES (pH 7.4), plus 1 mg/ml of collagenase, BSA and trypsin inhibitor. After incubation, each partially digested muscle layer was washed with enzyme-free HEPES medium, and incubated in fresh HEPES medium for 10 min. At the end of digestion, the muscular layer was dispersed into single cells with the assistance of gentle bubbling by pipette. The dispersed cells were then harvested by filtration through a 210-μm nylon mesh.

2.5. Measurement of the contractile response

The contractile response was measured in terms of the decrease in the average length of a population of SMCs exposed to various test agents.

Dispersed cells were stimulated by the addition of a given test agent to the cell suspension and then incubated at room temperature for 30 s. Each reaction was interrupted and fixed by the addition of acrolein to a final concentration of 1% (12), after which light microscopic images of each slide were captured on a computer. The length of approximately 50 cells on each slide was measured using Scion Image software (Scion Corporation, Frederick, MD, USA ver 4.0.3), after which the contractile response was assessed by measuring the decrease in average length of a population of smooth muscle cells exposed to a given test agent. CCh, an analog of the dominant excitatory neurotransmitter acetylcholine (ACh), was examined as the reference compound.

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