Effects of Papain on Isolation of Single Smooth Muscle Cells from the Guinea Pig Longitudinal Ileum

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Abstract

The methods for isolation of single cells from the guinea pig longitudinal ileum were investigated with focusing on the papain concentration for the digestion of the ileum. The ileal muscle was minced. The minced muscle was loaded with fluorescent probes of calcein or fura-2, and treated with papain for 30 min at various concentrations. Papain at concentrations more than 1 U/ml reduced both calcein fluorescence and fura-2-signal evoked by carbachol. Carbachol-induced fura-2-signal was more sensitive to papain than calcein fluorescence, suggesting that proteins related to the formation of receptors are more vulnerable than membrane lipids or proteins limiting the membrane permeability upon the exposure to papain. The resultant yield of single cells was highest at 0.56 U/ml of papain without affecting calcein and fura-2 fluorescence responses, thus this concentration appeared to be appropriate for the isolation of single cells from the ileum. Single cells alive contracted dose-dependently by the exposure to carbachol (0.1-10 μM) under the microscopic measurement, and were appeared to grow confluent in culture for approximately 15 days. These results suggest that the low concentration, 0.56 U/ml, of papain in the isolation medium is better to obtain functional cells from the guinea pig ileum.

Key words: single smooth muscle cells, cell viability, calcium signal, cell culture, ileal muscle.

Introduction

Smooth muscle responds to various stimuli, such as neurotransmitters, hormonal signals and so on. Although essential cellular components are shared by almost all smooth muscles, significant deviations in responsiveness to stimuli are observed among smooth muscles (Somlyo and Somlyo, 1994). The difference is more prominent when intestinal and vascular muscles are compared. Catecholamines like epinephrine often elicit relaxation in the intestine but contraction in the artery. To clarify the diversity of smooth muscles, single cells isolated from the responsible tissue are useful to understand the cellular basis of the regulatory mechanism.

The morphological organization is also different among smooth muscles, such as the...
various amount of extracellular matrix and the different interaction between cells. Thus, the method for isolation of smooth muscle cells may be different from tissue to tissue, when the functionally intact cells are required. Even in the intestinal smooth muscle of the same species, e.g., taenia coli and longitudinal ileum of the guinea pig, the methods for the isolation are slightly different with respect to the used concentration of papain and so on (cf. Maruyama et al., 1988 for the taenia, and Iijima et al., 1998 for the ileum). In comparison with many reported investigation using isolated cells from vascular tissue, the works using mammalian visceral smooth muscle were few (Sartore, et al., 1999). We have currently been preparing single cells from intestinal smooth muscle, and the amount of small intestine is rather abundant in the body. Thus, the intestine is a good source for obtaining single cells when the proper method is established. Here we described the rather detailed methods for isolation from the guinea pig longitudinal ileum, which will be useful for understanding the cellular function and the nature of differentiation and development of smooth muscle.

From our previous works on the isolation of single cells from smooth muscles (Maruyama et al., 1988; Fang et al., 1993; Iijima et al, 1998), the concentration of papain appears to be critical for the isolation, since this protease possesses a wide specificity to easily produce the denaturation of many proteins (Arnon, 1970). Thus, the present experiments have been focused on the effects of concentration of papain on the viability and Ca$^{2+}$-signaling at the minced muscle level, in addition to the yield of single cells. Some other critical procedures for the preparation of single cells and the subsequent primary culture have been also described.

Materials and methods

Preparation of longitudinal ileum

Male guinea pigs (Hartley, 250~500 g) were instantaneously killed by a blow on the head, and the small intestine was removed. Then, the strip of longitudinal ileum was prepared and placed in a physiological salt solution (PSS) composed of NaCl 137, KCl 2.7, CaCl$_2$ 1.8, MgCl$_2$ 1.0, glucose 5.6 and HEPES 4.2 (mM), pH 7.4, kept at 37°C for 60 min. The treatment of animals was approved by the Animal Welfare Committee of Showa University.

Chemicals and solutions used

Collagenase from clostridium histolyticam was the product of Amano Pharmaceutical Co. (Nagoya, Japan). Papain from papaya latex, minocycline, ampicillin and carbamylcholine chloride (carbachol) were obtained from Sigma Co. (St Louis, MO, USA). Bovine serum albumin (Fraction V), gentamicin, amphotericin B were the products of Boeringer Mannheim Co. (Germany). Following chemicals and solutions were used: Calcein/AM (Molecular Probe Inc. Eugene, OR, USA), fura-2/AM (Dojindo Laboratories, Kumamoto, Japan), minimum essential medium (MEM; Gibco BRL, Rockville, MD, USA) and fetal calf serum (FCS; Intergen Co., Purchase, NY, USA).

Preparation of single cells

Strips of longitudinal ileum were placed in the normal physiological salt solution for 60
Single smooth muscle cells from intestine

minces of longitudinal ileum in the Ca²⁺-free PSS

↓

enzyme treatment
(0.56 units of papain and 0.5 mg of collagenase per ml
in the presence of 1% BSA)

↓

incubated at 37°C for 30 min

↓

centrifuged at 400 rpm

↓

washed in the buffer

↓

dispersed by pipetting

↓

filtrated through nylon mesh (200 µm)

↓

centrifuged at 1,200 rpm for 5 min at 4°C

↓

single cells

Fig. 1. Diagram for the isolation of single cells from the longitudinal ileum of the guinea pig.

min, and were incubated in a Ca²⁺-free PSS at 37°C for 15 min. The Ca²⁺-free solution was made by the removal of CaCl₂ from the PSS.

Further procedure for preparing single cells is shown in Fig. 1. In the Ca²⁺-free PSS the strips were minced into small pieces by cutting the tissue with fine scissors. How many times of cutting was critical. For the ileal strip, 50 times cutting is much better to obtain good yield of cell number, compared with the excessive cutting of the tissue. On the other hand, for the guinea pig taenia coli, 500 times cutting was better.

The minced samples were treated for 30 min at 37°C with the mixture of various amount of papain, collagenase 0.5 mg and BSA 1% (final concentration). The enzyme-treated sample was suspended in the Ca²⁺-free PSS and centrifuged at 400 rpm for 2 min. The centrifugation was repeated three times for washing the isolated cells. The resultant pellet was gently dispersed into the Ca²⁺-free PSS with a wide bore pipette, then filtered through nylon mesh (200 µm). The filtrate was centrifuged at 1,200 rpm for 5 min at 4°C. The final pellet was gently dispersed in the Ca²⁺-free PSS at 4°C. This low temperature is critical to obtain single cells, since isolated cells often aggregated at 37°C. The obtained single cells were used for desired experiments.

The number of intact cells was counted using a phase contrast microscope. The cells not damaged morphologically were regarded as intact cells.
Primary culture of smooth muscle cells

Single smooth muscle cells prepared from the longitudinal ileum were suspended in MEM containing 20% FCS, 15 μg/ml gentamicin, 1 μg/ml minocycline, 2 μg/ml of amphotericin B and 50 μg/ml ampicillin. The cell suspension (7×10^3 cells/cm²) was seeded on a laminin-coated dish with 60 mm in diameter, and kept at 37°C under humidified conditions with 95% air and 5% CO₂. Twenty four hours after seeding, the culture medium was replaced with MEM containing 10% FCS, and the medium was changed every 3 days until cells were used for experiments.

The cells adhered to substrates were harvested and counted to estimate the cell number on the culture dish.

Calcein for the membrane permeability of cells in the minced smooth muscle

The fluorescent probe of calcein was used to investigate the effects of papain on the membrane permeability of smooth muscle minced with scissors as described above. The minced muscle was exposed to calcein/AM (4 μM) at 37°C for 30 min. After loading, the mince was washed with the Ca²⁺-free PSS and incubated in 1 ml of the Ca²⁺-free PSS with 0.5 mg collagenase and 1% BSA. Various concentrations of papain were administered and fluorescence of calcein was monitored for 30 min at 35°C with a fluorometer (excitation at 485 nm and emission at 530 nm: F-4000, Hitachi, Tokyo, Japan).

Fura-2 for cytosolic Ca²⁺ of the minced smooth muscle

The fluorescent probe of fura-2 was used to investigate the effects of papain on the cytosolic Ca²⁺ concentration of minced smooth muscle. The minced muscle was exposed to fura-2/AM (2 μM) at 37°C for 30 min. Then, the mince was washed with the Ca²⁺-free PSS and incubated in 1 ml of the Ca²⁺-contained. Various concentrations of papain were incubated for 30 min and carbachol (3 μM) was administered. Using a fluorometer (CAF-100, JASCO, Tokyo, Japan), fura-2 was excited with 340 and 380 nm, and their ratio of emission light at 500 nm was monitored at 20°C as the relative concentration of cytosolic Ca²⁺.

Dye exclusion test for cell viability

Trypan Blue dye exclusion test was applied for the isolated single cells to examine the cell viability according to Phillips (1973) with a lower concentration of the dye (final 0.1%).

Determination of cell contraction

Small amount of CaCl₂ (the final concentration of 1.8 mM) was added to the suspension of isolated single cells. The cell suspension was divided into several test tubes. After the equilibration for the desired period at 37°C, single concentration (0.1, 1 and 10 μM) of carbachol was added to each tube. Thirty seconds after the carbachol-addition, equal volume of 2% glutaraldehyde (pH 7.4) was added to the test tube for the fixation of single cells. Then, the longitudinal length of the fixed cells was determined with a computer-assisted light microscopic image analyzer (Olympus Optical Co., Tokyo, Japan). Decrease in the length (% decrease from the average length of control cells) was plotted against each concentration of carbachol.
Single cells not exposed to carbachol were served as control.

Results

Effects of papain on the plasma membrane permeability of the minced ileal muscle

When the plasma membrane becomes leaky, calcein entrapped by the cells in the tissue leaks out, resulting in the reduction of fluorescence of the cell or tissue. The longitudinal ileum of the guinea pig was minced and loaded with calcein. Fig. 2 shows the effects of papain on the calcein-fluorescence signal of the minced ileal muscle. In the control tissue without any treatment, the fluorescence intensity stayed at a relatively high level for 30 min, indicating that cells in the minced muscle keep the plasma membrane intact during the observation time. The exposure of the calcein-loaded muscle to 90% ethanol rapidly reduced the fluorescence due to the disruption of cell membrane. A treatment with the low concentration (2.8 U/ml) of papain did not reduce the fluorescence when compared with that of the control muscle. After the exposure to higher concentrations (14 and 70 U/ml) of papain, the fluorescence was gradually reduced and the reduction rate was dependent on the used concentration. The presence of papain (70 U/ml) for 30 min reduced the fluorescence by approximately 40% of the initial intensity before exposure to papain. These results suggest that the plasma membrane of cells in the minced ileal muscle becomes leaky upon the exposure to papain at concentrations over 2.8 U/ml.

![Graph showing the effects of papain on the membrane permeability of cells in the minced muscle](image)

**Fig. 2.** Effect of papain on the membrane permeability of cells in the minced muscle. Minces of longitudinal ileum were suspended in the Ca-free physiological salt solution (PSS) and incubated with calcein/AM. Then, enzyme treatment was carried out with various concentration of papain for 30 min. Fluorescence intensity was measured as described in the text. After the enzyme treatment, 90% ethanol was added to make cell membrane damaged thoroughly.
Effect of papain on Ca\textsuperscript{2+} signal from the minced ileal muscle

Fura-2/AM was loaded on the minced ileal muscle for the measurement of cytosolic Ca\textsuperscript{2+} concentration. The administration of carbachol (3 µM) promptly elevated the Ca\textsuperscript{2+} signal of the minced muscle. Fig. 3A shows the effect of papain treatment on the Ca\textsuperscript{2+} signal induced by carbachol. The carbachol-induced increase in Ca\textsuperscript{2+} signal was not affected by the presence of papain at 0.56 U/ml, but was inhibited by papain more than 1 U/ml. Papain at 14 U/ml

![Graph A](image1.png)

![Graph B](image2.png)

Fig. 3. Effects of papain on the Ca\textsuperscript{2+} response of cells in the minced muscle (A) and on the yield of single cells (B).

(A) Minces of longitudinal ileum were incubated in PSS with fura-2/AM, and various concentrations of papain were applied for 30 min. Then, carbachol (3 µM) was applied and fluorescence intensity was measured.

(B) Smooth muscle cells were isolated with various concentrations of papain, and the number of isolated cells was counted.
nearly abolished the Ca\textsuperscript{2+} signal. These results suggest that papain at concentrations more than 1 U/ml, but not at 0.56 U/ml, interfere with the cholinergic Ca\textsuperscript{2+} signaling of cells in the minced ileal muscle.

**Papain-concentration and number of single cells isolated from the ileal muscle**

To isolate single cells from the tissue, the longitudinal ileum was once minced and the minced muscle was exposed to various concentrations (0.14, 0.28, 0.56 and 1.4 U/ml) of papain. Fig. 3B shows the effect of the papain-concentration on the yield of single cells isolated from the ileal tissue. The yield of single cells from the tissue was maximum at 0.56 U/ml of the enzyme. Single smooth muscle cells prepared with 0.56 U/ml papain were spindle-shaped and approximately 100 \mu m in the longitudinal length (Fig. 4).

**Viability and contractility of single cells in the physiological salt solution**

Before commencement of experiments, isolated single cells were kept in the Ca\textsuperscript{2+}-free PSS at 4°C. Using Trypan Blue, the permeability of plasma membrane of isolated cells was tested every 30 min for 2 hours after the incubation of cells in the Ca\textsuperscript{2+}-free PSS (Fig. 5). Promptly after single cells were prepared (at 0 time), the plasma membrane of about 20% of dispersed cells was permeable for Trypan Blue. Gradually more cells became permeable for the dye, then, 90–120 min after incubation the nearly steady conditions were obtained. In terms of the membrane permeability, approximately half of originally dispersed cells were viable for 120 min in the Ca\textsuperscript{2+}-free PSS.

To test the contractility single cells were incubated in the normal PSS at 37°C. When carbachol (0.1–10 \mu M) was administered, the longitudinal length of single cell rapidly decreased in 30 sec. The decrease in length depended on the concentration of carbachol (Fig. 6). Upon the exposure to 10 \mu M carbachol, the decrease in length appeared to be about 40% of the
Fig. 5. Viability of single cells after isolation. Isolated cells were suspended in the Ca\(^{2+}\)-free PSS and kept at 4°C. Every 30 min cell viability was measured by the trypan blue dye exclusion test.

Fig. 6. Relationship between longitudinal length of single cells and concentration of carbachol. Cells were incubated with various concentration of carbachol for 30 sec at 37°C. After mixing with glutaraldehyde solution, the cells were transferred to a microscopic slide, and their dimensions were recorded using a computer-assisted image analyzer. These results indicate that the single cells isolated through the present method maintain contractility.
Primary culture of single smooth muscle cells

The obtained single cells were dispersed in the culture medium and were seeded in culture dishes with or without the coat of substrate such as laminin, collagen and fibronectin. When dishes without coating were used, cells grew very slowly. In dishes coated with collagen or fibronectin, the reproducible growth of cells could not be obtained during culture. Finally, laminin-coated dishes appeared to be appropriate for the primary culture of single cells prepared from the ileal longitudinal smooth muscle.

The number of cells after the primary culture of single cells was counted to assess the cell growth (Fig. 7). The number of cells markedly decreased 1 and 2 days after seeding the single cells, indicating that approximately 5% of seeded cells were attached to the laminin-coat on the dish. At day 3 of culture the cell number began to increase, and the gradual increase was observed 6-9 days after seeding. Then, the confluent stage of culture was attained 12 days after seeding.

Fig. 8 shows photographs of cells taken at the day 1, 4, 9 and 16 of culture. At the day 1 of culture, spindle shaped cells and round-shaped cells were observed: the number of round cells exceeded that of spindle cells. At day 3, a network-like structure was observed between cells. This network structure seemed to be made by protrusions from cells. Nine days after culture, cells proliferated with increasing the cell number. Confluently grown cells (day 16) formed the pattern of "hill and valley", as reported in other smooth muscle cell culture (Gordon et al., 1986; Ma et al., 1998).
Fig. 8. Changes in culture of smooth muscle cells isolated from guinea pig ileum. Phase contrast micrographs were taken on days 1, 3, 9 and 16 of culture.

Discussion

Previously, we obtained single cells from taenia coli of the guinea pig and those single cells contracted upon the exposure to carbachol (Maruyama, et al., 1988). In contrast, present experiments demonstrate that the methods for the taenia coli do not yield single cells with respect to the membrane permeability and cell contractility. There are at least two critical factors in the methods of single cell preparation. One is the method for mincing the tissue. Compared with the taenia coli, one tenth of the number of mincing times, only 50 times, appeared to be appropriate for obtaining single cells in terms of the yielded cell number and cell responsibility to carbachol. The other crucial factor is the treatment of the minced ileum with papain. The concentration of papain appeared to be lower for the preparation from the ileum than for that from the taenia coli. Considering with the Ca$^{2+}$-signal response to carbachol and membrane permeability of the minced ileum as well as the yielded number and contractility of single cells, the proper concentration for the isolation was determined as 0.56 U/ml among examined concentrations of 0.14–70 U/ml. The determined concentration is 1/5 of that for the taenia coli. These results suggest that the milder conditions are required for the isolation of single cells from the longitudinal ileum than that from the taenia coli of the guinea pig. Presumably, the different conditions of the methods reflect the difference in morphology and constituents between two intestinal smooth muscles of ileum and taenia coli of the same species, although the exact nature of differences is not known at the present moment.
Measurements of fluorescences from fura-2 and calcein entrapped in the minced ileum revealed that high concentrations of papain (more than 2-3 U/ml) impaired the cellular Ca\textsuperscript{2+}-response to carbachol and the plasma membrane permeability, respectively. Exposure to 2.8 U/ml papain inhibited the carbachol-induced Ca\textsuperscript{2+}-signal only by 20% of control, but not affect the membrane permeability. In the previous our work, the binding assay using the single cells from the ileum showed that Kd values for cholinergic drugs were not changed by the exposure to papain 2.8 U/ml (Fang et al., 1993). Therefore, the damage due to the treatment with 2.8 U/ml papain seems to be small. When papain at 14U/ml was exposed, the calcein fluorescence was lost by 15% of control, whereas, the carbachol-induced cellular Ca\textsuperscript{2+}-signal was nearly abolished. These results suggest that, upon the exposure to papain, the cellular Ca\textsuperscript{2+}-response to carbachol was much more vulnerable than the membrane permeability. Papain is a protease, so that the receptor proteins or their accessory proteins may be more sensitive to papain than the membrane lipids or proteins which limit the permeability against large molecules like calcein.

The single cells isolated from the guinea pig ileum through the present methods were still deteriorated gradually but half or more of the initially prepared cells were usable for experiments at least until 2 hours after isolation. When single cells were subsequently cultured, the viable and proliferatable cells were only 5% of the number of seeded cells. Presumably, the antibiotic, amphotericin B, present in the initial medium for culture destroys many cells to death. The residual cells started to grow up the confluent stage. However, the grown cells after culture did not possess the original phenotype. Cultured cells lost contractility and altered isoenzyme patterns, but could produce the Ca\textsuperscript{2+}-signal response to carbachol, as reported previously (Iijima et al., 1998). The reason of alteration from smooth muscle phenotype to the non-muscle phenotype during culture is the subject for the future study.

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(Received July 12, 1999: Accepted September 29, 1999)