Characteristics of subepithelial fibroblasts as a mechano-sensor in the intestine: cell-shape-dependent ATP release and P2Y1 signaling

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Summary

Subepithelial fibroblasts form a cellular network just under the epithelium of the gastrointestinal tract. Using primary cultured cells isolated from rat duodenal villi, we previously found that subepithelial fibroblasts reversibly changed cell morphology between flat and stellate-shape depending on intracellular cAMP levels. In this paper, we examined cell-cell communication via released ATP and Ca^{2+} signaling in the cellular network. Subepithelial fibroblasts were sensitive to mechanical stress such as 'touching' a cell with a fine glass rod and 'stretching' cells cultured on elastic silicone chamber. Mechanical stimulations evoked Ca^{2+}-increase in the cells and ATP-release from the cells. The released ATP activated P2Y receptors on the surrounding cells and propagated Ca^{2+}-waves through the network. Concomitant with Ca^{2+}-waves, a transient contraction of the network was observed. Histochemical, RT-PCR, western blotting and Ca^{2+} response analyses indicated P2Y1 is a dominant functional subtype. ATP-release and Ca^{2+} signaling were cell-shape dependent, i.e. they were abolished in stellate-shaped cells treated with dBcAMP, and recovered or further enhanced in re-flattened cells treated with endothelin. The response to ATP also decreased in stellate-shaped cells. These findings indicate cAMP-mediated intracellular signaling causes cell-shape change, which accompanies the changes in mechano- and ATP sensitivities. Using a co-culture system of neuronal cells (NG108-15) with subepithelial fibroblasts, we confirmed that mechanically induced Ca^{2+}-waves propagated to neurons. From these findings we propose that subepithelial fibroblasts work as a mechano-sensor in the intestine. Uptake of food, water and nutrients may cause mechanical stress on subepithelial fibroblasts in the villi. The ATP released by mechanical stimulation elicits Ca^{2+}-wave propagation through the network via P2Y1 activation and also activates P2X on terminals of mucosal sensory neurons to regulate peristaltic motility.

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Key words: Subepithelial fibroblasts, Mechanosensing, Intestine, ATP release, P2Y, Myofibroblasts

Introduction

Under the epithelium of the gastrointestinal tract, subepithelial fibroblasts exist as a cellular network from the esophagus to the anus. They have flattened cell bodies with numerous long, attenuated cell processes, are rich in α-smooth muscle actin (α-SMA), communicate via gap junctions (Güldner et al., 1972; Desaki et al., 1984; Joyce et al., 1987; Komuro and Hashimoto, 1990), and their cellular network ensheaths the lamina propria of intestinal villi, just like a nylon stocking. Moreover, subepithelial fibroblasts are connected with α-SMA-negative fibroblast-like cells in the lamina propria, forming a three-dimensional cellular network (Joyce et al., 1987; Adegboyega et al., 2002). These subepithelial fibroblasts and fibroblast-like cells are in close contact with nerve terminals, venous capillaries and smooth muscles (Güldner et al., 1972; Desaki et al., 1984; Joyce et al., 1987). It is now being recognized that subepithelial fibroblasts not only secrete components of the basal lamina, but also play major roles in the regulation of proliferation, migration, transepithelial resistance and secretory responses of epithelial cells, and in the regulation of inflammatory and injury responses in the gut (Powell et al., 1999a; Powell et al., 1999b).

We found that subepithelial fibroblasts of rat small intestinal villi are rich in endothelin (ET) receptors by electron microscopic autoradiography and immunohistochemistry (Furuya et al., 1990; Furuya et al., 1991; Furuya et al., 2005). By the establishment of a primary culture system from rat duodenum villi (Furuya and Furuya, 1993), we found rapid cell-shape changes from flat morphology with broad cell processes (flat-shape) to round cell body with thin cell processes (stellate-shape) by dibutyryl cyclic AMP (dBcAMP), forskolin and cholera toxin, and reversed morphological changes from stellate to flat upon the addition of serum and ETs, just as reported in cultured astrocytes (Goldman and Abramson, 1990). Subepithelial fibroblasts express many kinds

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of receptors, such as ETs, angiotensin II, ATP, ADP, bradykinin, serotonin, substance P (Furuya et al., 1994). In contrast with cultured astrocytes, subepithelial fibroblasts are rich in endothelin A (ET_A) receptors, and gap junctions are commonly opened in a cAMP-independent manner, i.e. independent of cell-shape changes (Furuya et al., 2005). We have preliminarily reported that subepithelial fibroblasts are sensitive to mechanical stimulation and communicate not only via gap junctions, but also via humoral factors, especially ATP (Furuya and Furuya, 2002). From the anatomical location and cellular characteristics, we believe that subepithelial fibroblasts play a key role in the intercellular signal transduction in the villi, which may be modulated by cell morphological changes.

The gastrointestinal tract is not only regarded as a digestive and an immune organ but also as a sensory organ (Furness et al., 1999). Food and water uptake and their digestion may give rise to chemical and mechanical signals that induce the peristaltic reflex in the gut. Chemical and mechanical signals control motility, secretion and immune defenses in local and/or central neural and/or non-neural pathways (Buchan, 1999; Furness et al., 1999; Furness et al., 2004). It has been suggested that nutrients in the lumen evoke cholecystokinin (CCK), serotonin and ATP release from enteroendocrine and enterochromaffin cells located in epithelia, and these substances act as sensory mediators for sensory neurons (Eastwood et al., 1998; Buchan, 1999; Höfer et al., 1999).

ATP and nucleotides are now recognized as important and ubiquitous extracellular messengers in various kinds of tissues and organs (Abbracchio and Williams, 2001; Burnstock and Knight, 2004). ATP is often released by mechanical stimulations and activates surrounding cells via many subtypes of P2Y metabotropic and P2X ionotropic ATP receptors (Schwiebert, 2000). The released ATP works as autocrine and paracrine mediators, and play pivotal roles in the mechano-transduction in many tissues and organs, such as blood vessel endothelium (Burnstock, 1999; Yamamoto et al., 2000), airway epithelium (Hansen et al., 1993; Homolya et al., 2000), mammary epithelial cells (Enomoto et al., 1994; Furuya et al., 1997; Nakano et al., 1997), urinary bladder epithelium (Cockayne et al., 2000; Vlaskovska et al., 2001), intestine (Burnstock, 2001b; Wynn et al., 2003), liver epithelial cells (Frame and de Feijter, 1997), osteoblasts (Jørgensen et al., 1997), keratinocytes (Denda et al., 2002; Koizumi et al., 2004), pancreatic islet cells (Cao et al., 1997), mast cells (Osipchuk and Cahalan, 1992) and astrocytes (John et al., 1999; Cotrina et al., 2000; Fam et al., 2000; Scemes et al., 2000; Newman, 2001; Koizumi et al., 2003). In blood vessels, blood flow induces ATP release from endothelial cells (Burnstock, 1999) and ATP enhances mechano-sensitivity (Yamamoto et al., 2000). In airways, ciliated epithelial cells release ATP after mechanical stress induced by foreign substances, and enhance salt and water transport, ciliary beat frequency and mucin secretion to increase defense mechanisms (Hansen et al., 1993; Homolya et al., 2000). In mammary alveoli, myo- and secretory epithelial cells interact mutually via mechanically released ATP to enhance milk secretion (Enomoto et al., 1994; Furuya et al., 1997; Nakano et al., 1997).

It has been proposed that in tubular and bladder visceral organs, e.g. intestine (Burnstock, 2001b; Wynn et al., 2003) and urinary bladder (Cockayne et al., 2000; Vlaskovska et al., 2001), nociceptive mechano-sensory transduction occurs by ATP released from epithelium by the distension or distortion of these organs, which then activates P2X3 and/or P2X2 or P2X2/3 receptors on subepithelial sensory nerve plexuses to relay messages to the CNS pain centers (Burnstock, 2001a; Cooke et al., 2003; Wynn et al., 2003). However, what type of cells in the epithelium are really responsible for ATP-release or for working as mechano-sensors is still not clear, although, in many cases, epithelial cells are thought to release ATP by mechanical stress.

In this paper, we report that mechanical stimulations (touching and stretching) to the cellular network of subepithelial fibroblasts in intestinal villi evoke cell-shape-dependent ATP release and Ca^{2+} wave propagation via P2Y1 receptors. The location and characteristics of subepithelial fibroblasts clearly indicate that they may work as the mechano-sensor in the intestinal villi. To confirm this hypothesis we demonstrate the transduction of Ca^{2+} signaling from subepithelial fibroblasts to neuronal cells in a co-cultured system. A preliminary report of this work appeared in an abstract form (Furuya and Furuya, 2002).

Materials and Methods

Animals

Wild-phenotype congenital aganglionosis rats (AR) (Karaki et al., 1996; Kunieda et al., 1996; Shim et al., 1996) used for this study were maintained in the animal center of the National Institute for Physiological Sciences. Wistar rats were obtained from Japan SLC Inc. (Hamamatsu, Japan). All protocols were approved by the Animal Use Committee of Okazaki National Institutes and follow the guidelines of animal care and experiments of the National Institute for Physiological Sciences.

Cell cultures

Subepithelial fibroblasts

Subepithelial fibroblasts were cultured as previously described (Furuya and Furuya, 1993). Briefly, villi isolated from 10-12-day-old wild-type rat duodenum were incubated with Dulbecco's modified Ca^{2+}, Mg^{2+}-free phosphate buffered saline supplemented with 1 g per litre glucose for 30 minutes with shaking at 75 rpm, and were vortexed for 30 seconds to remove epithelial cells from the villi. Epithelium-free duodenal villi were re-suspended in DMEM/F12 containing 10% FCS, 50 μg ml^{-1} streptomycin and 50 U ml^{-1} penicillin, and inoculated on collagen-coated 15 mm round coverslips or elastic chambers made from silicone elastomer resins, and incubated at 37°C in an atmosphere of 5% CO_2/95% air at saturated humidity for 3 days. Subepithelial fibroblasts migrated towards the exterior as a monolayer of maple leaf-like cells.

18Co cells

The CCD-18Co cell line derived from human colon (CRL-1459, ATCC) is known to express similar characteristics of subepithelial fibroblasts (Valentich et al., 1997). Cells were cultured to confluence with DMEM containing 10% FCS.

Co-culture of subepithelial fibroblasts and NG108-15 cells

NG108-15 cells were differentiated for 5-7 days in DMEM containing 3% FCS, HAT supplements and 1 mM dBcAMP (Nelson et al., 1976; Hamprecht, 1977; Furuya and Furuya, 1983). Dissociated cells were

inoculated onto coverslips where subepithelial fibroblasts were pre-cultured for 3 days. Subepithelial fibroblasts and NG108-15 cells were co-cultured for several hours or overnight with DMEM/F12 containing 10% FCS.

Immunohistochemistry

Rat small intestines

Wild-type AR rats and Wistar rats were perfused with 4% paraformaldehyde/0.2% picric acid in 0.1 M phosphate buffer pH 7.4, and processed for light microscopic immunohistochemistry as previously described (Furuya et al., 2001). 10 μm thick cryosections were incubated with methanol containing 0.3% H_2O_2 for 30 minutes to inhibit endogenous peroxidase activity, and incubated with 10% NGS in PBS for 30 minutes. Cryosections were incubated with rabbit anti-P2Y1 antibody (3 μg ml^{-1}), rabbit anti-P2Y2 antibody (1:200 dilution) or rabbit anti-P2Y4 antibody (1.5 μg ml^{-1}) overnight at 4°C, followed by anti-rabbit labeled Polymer, HRP (ENVISION™ + system) for 2 hours at room temperature, and finally reacted with 0.02% DAB-0.01% H_2O_2 in 0.05 M Tris-HCl (pH 7.6) for 5 minutes. After dehydration with ethanol followed by xylene, sections were mounted and examined under an upright microscope (Olympus AX70, Tokyo).

Cultured subepithelial fibroblasts

Subepithelial fibroblasts cultured on 15 mm coverslips for 3 days were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 for 30 minutes. After incubation with 10% NGS containing 0.1% triton X for 30 minutes, cells were incubated with rabbit anti-P2Y1 antibody (3 μg ml^{-1}) in 1% BSA containing 0.1% triton X overnight at 4°C, and then incubated with biotin-conjugated anti-rabbit IgG (5 μg ml^{-1}) for 2 hours, followed by streptavidin Texas red (1:200 dilution in PBS) for 2 hours at room temperature. After washing with PBS, cells on the coverslips were mounted upside down onto slide glasses with Perma Fluor aqueous mounting medium and observed under a fluorescence microscope (Olympus BX).

Pre-absorption of anti-P2Y1antibody

Synthetic peptide corresponding to residues (242-258) of rat P2Y1 was added at various concentrations (0, 2, 5 and 10 μg ml^{-1}) to anti-P2Y1 antibody (3 μg ml^{-1}) in PBS containing 1% BSA-0.05% NaN_3 and incubated overnight at 4°C. After centrifugation at 10,000 ***g*** for 20 minutes, supernatants were used for pre-absorption tests.

RT-PCR analysis

Cultured cells were lysed by addition of RNA STAT-60 directly onto culture dishes (1 ml per 35 mm plastic dish) and total cellular RNA was prepared according to the manufacturer's instructions. Glycogen (200 μg) was added to cell lysates as a carrier to co-precipitate a small amount of nucleotides. Precipitated nucleic acids were washed twice in 70% ethanol, dried, and re-suspended in water. Single-stranded cDNAs were synthesized using SuperScript™ III Reverse Transcriptase in a reaction mixture of 3 μg of RNA and 25 pM of random primers according to the manufacturer's protocol. One-tenth volume of cDNA was amplified using Ex Taq™ DNA polymerase, and sense and antisense oligonucleotide primers (see supplementary material, Table 1) with a DNA Thermal Cycler, Perkin-Elmer 480, according to the manufacturer's protocol. PCR was performed for 30 cycles under the following conditions: 30 seconds at 94°C for denaturation; 1 minute at 54, 55 or 57°C for annealing; 1 minute at 72°C for elongation. After the 30th cycle, elongation was terminated at 72°C for 7 minutes. PCR products were separated on 2% agarose gels in the presence of ethidium bromide.

Western blot analysis

Subepithelial fibroblasts and 18Co cells were cultured in 35 mm plastic dishes for 3 days and solubilized with 1×SDS sample buffer (2% SDS, 50 mM Tris-HCl, pH 6.8, 6% β-mercaptoethanol, 10% glycerol). Cerebra isolated from 3-week-old rats were homogenized on ice using a homogenizer (Polytron RT1200) in 3 volumes of PBS containing protease inhibitors (0.1 mM APMSF, 10 μg ml^{-1} leupeptin and 10 μg ml^{-1} pepstatin), and centrifuged at 7500 ***g*** for 10 minutes at 4°C. Pellets were resuspended in the above buffer, and then same volume of 2×SDS sample buffer was added to solubilized homogenates. Each sample was boiled for 2 minutes and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) performed by the method of Laemmli (Laemmli, 1970) using 10% polyacrylamide gels. SDS-PAGE-separated specimens were transferred onto blotting membranes (Immobilon-P, Millipore). Membranes were blocked with 5% skimmed milk (Difco) for 30 minutes, and incubated with rabbit anti-P2Y1antiboy (3 μg ml^{-1} in PBS containing 1% BSA) overnight at 4°C, followed by anti-rabbit-labeled Polymer, HRP antibody (ENVISION™ + system, 1:4 dilution in PBS) for 2 hours at room temperature. Finally, membranes were incubated for 1 minute with Western Blot Chemiluminescence Reagent Plus (Perkin-Elmer Life Sciences, Inc., Boston), and exposed on X-ray films (Konica Medical Film, Konica, Tokyo).

Mechanical stimulations

'Touching'

A single cell was stimulated by slight touching with a glass microelectrode, whose tip had been heat blunted. A piezo-driven micromanipulator (Burleigh PZ-301) was used to move the glass rod. To touch the cell without detectable damage, Z-axis movement of the manipulator was controlled by an input voltage generated by a function generator.

'Stretching'

An elastic chamber was made with silicone elastomer (Sylgard 184, Dow Corning, Midland, USA) and cells were cultured on chambers coated with collagen. The chamber was set on a pulse-motor-driven stretch machine (Naruse et al., 1998) (STREX, Osaka, Japan), and stretched 5-60% (length) for 2-3 seconds during each stimulus.

Measurements of released ATP

In some experiments, perfusates from elastic chambers (0.2-0.5 ml per minute) were collected every minute and ATP contents in perfusates were measured by a luciferin-luciferase bioluminescence reagent (Lucifere250 Kikkoman, Noda, Japan) that included luciferin and recombinant-luciferase in a buffered solution. 25 μl of perfusate and reagent were mixed in a plastic test tube and set in a luminometer (Berthold LB9506) to measure luminescence for 15-30 seconds. ALU (arbitrary light units) were converted to ATP by calibrating ATP solutions of known concentration. In this particular primary culture, it was difficult to inoculate an equal number of subepithelial fibroblasts or to measure accurately the number of subepithelial fibroblasts in culture. So, the ATP concentration varied from preparation to preparation. For this reason, trace data were shown only by typical data, and averaging was done after normalization with each control experiment.

Measurements of intracellular Ca^{2+} changes

To load a fluorescence calcium indicator, cells were incubated with 2 μM indo-1 AM and 0.2% cremophor EL in culture media for 1 hour at 37°C. Cells on the coverslip were placed in a perfusion chamber and cells on elastic chambers were set on a stretch machine. They were mounted on the stage of an inverted microscope (Zeiss Axiovert

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135) and were perfused continuously with DME (Sigma) buffered with 10 mM HEPES (pH 7.4), or normal Ringer's solution consisting of mM amounts of: NaCl 152, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.8, glucose 5.6, HEPES 10 (pH 7.4). Ca^{2+} was depleted from the solution by adding EGTA (10 mM) to HEPES-buffered DME or by using a Ca^{2+} free solution consisting of mM amounts of: NaCl 152, KCl 5.4, $MgCl_2$ 5.0, EGTA 2, glucose 5.6 and HEPES 10 (pH 7.4).

Calcium changes within cells were imaged with a laser scanning confocal microscope (Zeiss LSM410) equipped with a UV laser (Spectra-physics 2017-06). Indo-1 was excited at 360 nm and fluorescence emission was observed at 405 nm and 480 nm (F405, F480). Simultaneously, transmitted Nomarski images were observed with a 633 nm He-Ne laser. Intracellular Ca^{2+} changes were displayed by superposition of these three images in RGB pseudo color (red is F405, green is F480 and blue is Nomarski images), or ratio images calculated by (F405–b)/(F480×a), where a and b are constants (a is 128-160, b is 10-20). Experiments were performed at room temperature (24±2°C).

Materials

Dibutyryl cyclic AMP (dBcAMP): Yamasa, Chiba, Japan; endothelin 1 (ET1), substance-P: Peptide Institute, Osaka, Japan; ATP, UTP, ADP, UDP: Yamasa and Sigma, St Louis, MO, USA; 2MeSATP, MRS2179, MRS2159, carbenoxolone disodium salt (CBX): Sigma, suramin hexasodium: RBI, Natick, MA, USA; carbachol: Calbiochem, San Diego, CA, USA; Indo-1AM: Dojindo, Komamoto, Japan; cremophor EL: Sigma; Cellmatrix type I-A (Collagen): Nitta, Osaka, Japan; Biotin-conjugated goat anti-rabbit IgG (minimal cross-reaction): Jackson Immuno Res Labs, West Grove, PA, USA; streptavidin-Texas Red: Amersham, Piscataway, NJ, USA; rabbit anti P2Y1 receptor antibody, synthetic peptide (242-258) of rat P2Y1, rabbit anti P2Y4 receptor antibody: Alomone Labs Ltd, Jerusalem, Israel; rabbit anti P2Y2 antibody: Neuromics Inc., Minneapolis; ENVISION™ + system: Dako, Carpinteria, CA, USA; PermaFluor aqueous mounting medium: Thermo/Shandon Immunon, Pittsburgh, PA, USA; RNA STAT-60: TEL-TEST, INC. Friendswood, TX, USA; Ex Taq™: TaKaRa BIO INC, Otsu, Japan; SuperScript™ III Reverse Transcriptase: Invitrogen, Carlsbad, CA, USA.

Results

Subtypes of ATP receptors in subepithelial fibroblasts

Immunohistochemistry of P2Y in the rat small intestine and cultured subepithelial fibroblasts

P2Y1 immunoreactivity was observed just in the lamina propria and submucosa of rat small intestine (Fig. 1A). Immunoreactivity was completely abolished by the absorption of an antiserum with 2 μg ml^{-1} of antigenic synthetic peptide (Fig. 1B). P2Y2 and P2Y4 immunoreactivity was not observed in the small intestine (data not shown). Significant differences were not observed between wild-type AR and Wistar rats. In subepithelial fibroblasts cultured for 3 days, P2Y1 immunofluorescence was distributed throughout the cells (Fig. 1C).

RT-PCR analysis

Subtypes of ATP receptors in primary cultured subepithelial fibroblasts isolated from duodenal villi were examined by RT-PCR analysis (Fig. 2A). P2Y1 and P2Y4 mRNA were dominantly expressed in every culture from controls (flat-shaped cells, *n*=6 cultures) and stellate-shaped cells treated with dBcAMP (*n*=3 cultures, data not shown). P2Y2 and P2X1 mRNA were sometimes detected, although these bands were faint. P2Y6, P2Y12, P2X2 and P2X7 mRNA were not detected (not shown). Significant differences were not observed between control and stellate-shaped cells treated with 1 mM dBcAMP for 4-6 hours (data not shown).

In the primary culture, contamination by other cell types cannot be excluded. Subepithelial fibroblasts were identified by their ability to change cell shape from flat to stellate by dBcAMP or forskolin treatment (Furuya and Furuya, 1993). Using this criterion, our culture was estimated to contain about 90% of subepithelial fibroblasts.

In the CCD-18Co cell-line derived from human colon, there should be no such contamination of other cell types. RT-PCR analysis of 18Co cells showed abundant P2Y1 mRNA, and scarce P2Y4 mRNA (Fig. 2B). P2Y2 and P2X1 were not detected at all. Expression of P2Y1 seemed to be a common characteristic of subepithelial fibroblasts.

Western blot analysis

To confirm our result, we checked the expression of P2Y1 protein by western blotting analysis (Fig. 2C). Anti-P2Y1 antibody detected a band at approximately 70 kDa in the lysate of cultured subepithelial fibroblasts (column A) and 18Co cells (column C). A positive control consisting of rat

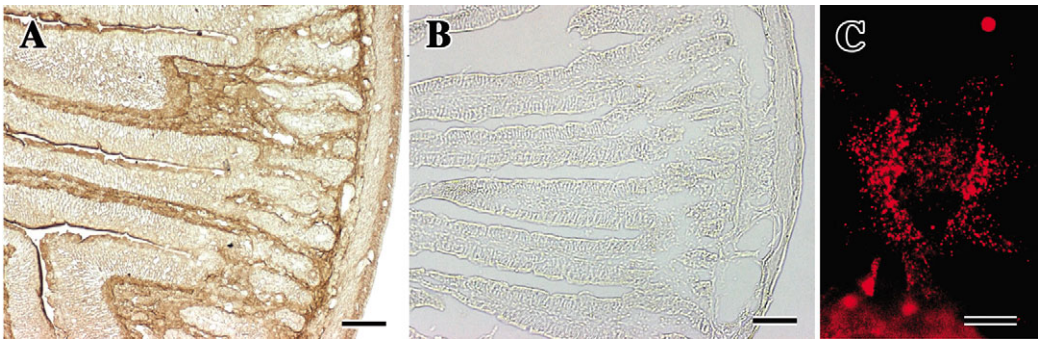

Fig. 1. Localization of P2Y1 in rat duodenum and in cultured subepithelial fibroblasts. Cryosections of 6W Wistar rat duodenum were incubated with (A) rabbit anti-P2Y1 antibody and (B) anti-P2Y1 antibody pre-absorbed with 2 μg ml^{-1} antigenic peptide, followed with anti-rabbit Envision+ and visualized with DAB-H_2O_2 reactions. (C) Subepithelial fibroblasts isolated from rat duodenal villi in primary cultures were incubated with rabbit anti-P2Y1 antibody, then biotinylated anti-rabbit IgG, followed with streptavidin-Texas red. Bar in A and B is 100 μm, and in C is 10 μm.

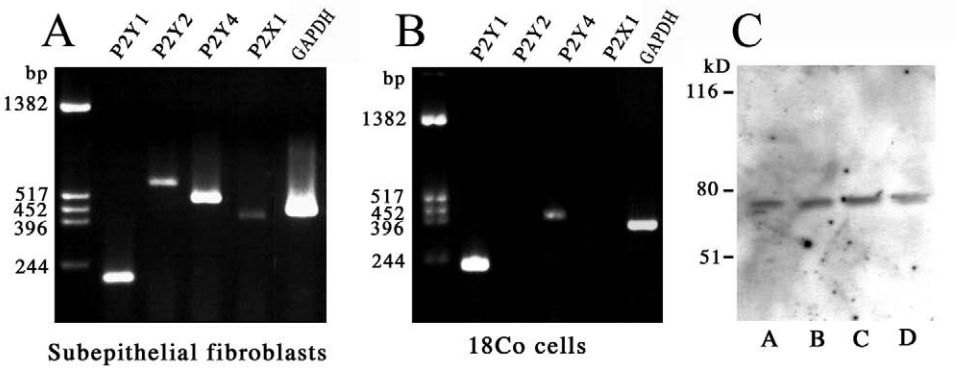

Fig. 2. Expression of P2Y1 mRNA and P2Y1 protein. RT-PCR analysis of subtypes of ATP receptors (A) in the primary culture of subepithelial fibroblasts and (B) in 18Co cells. (C) Western blotting analysis of P2Y1 receptors. Samples were loaded as follows. Lane A: subepithelial fibroblasts cultured in medium containing 10% FCS (control) (20 μg per lane). Lane B: subepithelial fibroblasts incubated in medium with 1 mM dBcAMP without FCS for 2 hours (20 μg per lane). Lane C: 18Co cells (50 μg per lane). Lane D: 3-week-old rat cerebrum (100 μg per lane). Samples were run on 10% SDS-polyacrylamide gels, transferred onto blotting membranes and detected with anti-P2Y1 antibody.

cerebellum lysate contained a similar sized band (column D) so the band should correspond to P2Y1, even though the estimated size of this protein is 42 kDa. There were no obvious differences in the amount and position of P2Y1 bands between control (flat-shape) and stellate-shaped cells treated with 1 mM dBcAMP for 2 hours (column B). In the RT-PCR analysis, P2Y4 mRNA was detected in some cases, but P2Y4 protein could not be detected by western blot analysis (data not shown).

Functional analysis using Ca^{2+} responses to nucleotides

The application of ATP (0.1-100 μM) evoked transient increases in intracellular Ca^{2+} in cultured subepithelial fibroblasts (Fig. 3A). ADP induced an intense response similar to ATP, but UTP elicited a lesser response and UDP had almost no response (Fig. 3A). For the first time, Ca^{2+} responses were observed even in Ca^{2+}-depleted medium (Fig. 3B). The potency of these nucleotides was ADP≥ATP>>UTP>UDP, suggesting

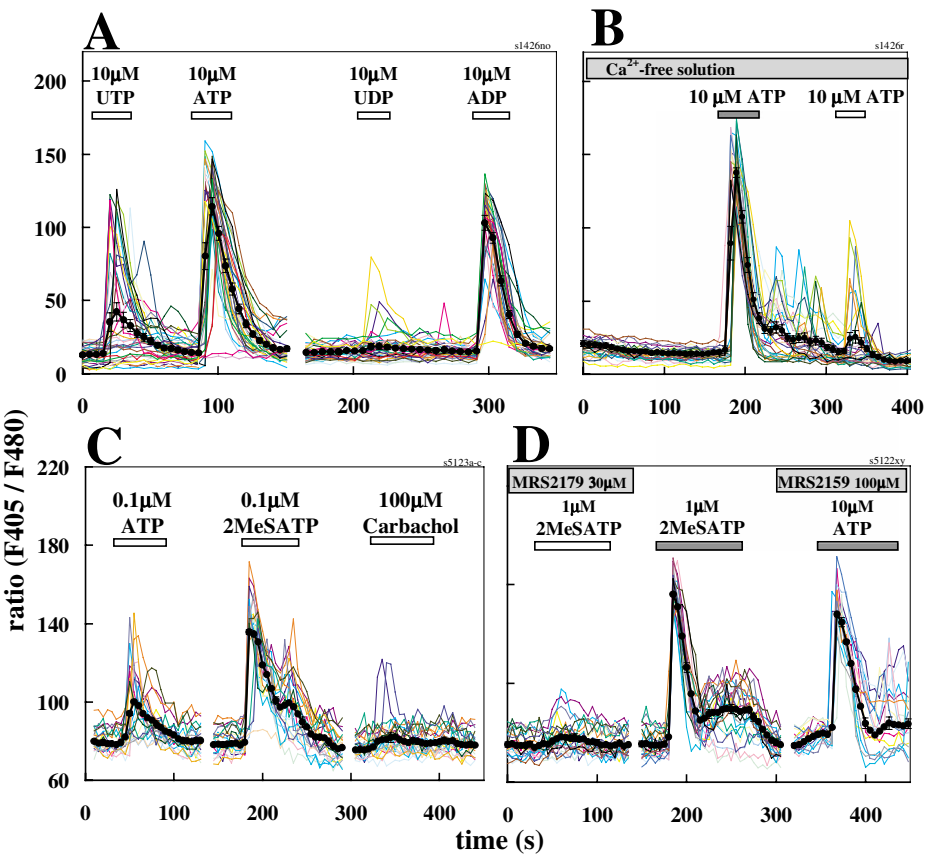

Fig. 3. The effect of agonists and antagonists of ATP receptors on Ca^{2+} responses. Intracellular Ca^{2+} responses shown by indo-1 fluorescence ratios (F405/F480), were measured in subepithelial fibroblasts cultured for 3 days using a laser confocal microscope. Color traces show responses in each cell and black traces with filled circles indicate the average response. (A) Ca^{2+} responses to UTP, ATP, UDP and ADP. The nucleotide concentrations were 10 μM. ATP and ADP are equally potent to induce Ca^{2+} increase, UTP is less effective, and UDP is not effective at 10 μM. (B) In Ca^{2+}-free solution, a similar Ca^{2+} response to ATP was initially observed, but later was suppressed. (C) 2MeSATP, a P2Y1 agonist, is more effective than ATP. The responses to each agonist at concentration 0.1 μM are compared. In the same cells, carbachol, a cholinergic agonist, is not effective even at 100 μM. (D) MRS2179 (30 μM), a P2Y1 antagonist, potently blocks the Ca^{2+} response to 2MeSATP (1 μM). The response is recovered after 10 minutes washout of MRS2179. Conversely, MRS2159 (100 μM), a P2X1 antagonist, is not effective on the Ca^{2+} response.

a contribution of P2Y1 receptors. 2MeSATP, which acts specifically on P2Y1 from the P2Y family, was more potent than ATP (Fig. 3C). MRS2179, a P2Y1 antagonist, blocked Ca^{2+} responses to 2MeSATP, but MRS2159, a P2X1 antagonist, had no effect (Fig. 3D). These pharmacological data support the idea that P2Y1 is a dominantly expressed ATP receptor in subepithelial fibroblasts.

Mechano-sensitivities of subepithelial fibroblasts

Touch-induced intercellular Ca^{2+} waves

The touching of a subepithelial fibroblast in control cultures (flat-shaped cell with broad several cell processes) with a blunted thin glass rod induced an intracellular Ca^{2+} increase in the cells and the Ca^{2+} increase propagated to surrounding cells in a wave-like manner (intercellular Ca^{2+} wave) (Fig. 4A, supplementary material Movie 1). Ca^{2+} waves propagated 150-200 μm in radius with a speed of 5-10 μm per second. Ca^{2+} wave propagations were reversibly blocked by MRS2179 (100 μM), an inhibitor of P2Y1 receptors, (Fig. 4B) and by suramin, an inhibitor of P2Y, and by apyrase (data not shown), but not affected by a gap junction blocker, CBX (100 μM) (Fig. 4C). Ca^{2+} waves propagated to separate cells where no physical contact existed between the cells (Fig. 4D). The touching of the cells in Ca^{2+}-free solution also induced Ca^{2+} increases in the cells and the Ca^{2+} waves in surrounding cells during an initial stimulation (Fig. 4E,F). These results strongly suggest that touch induced a release of active substances, such as nucleotides, from stimulated cells, which activated P2Y1 receptors in surrounding cells. These processes formed propagating intercellular Ca^{2+} waves in subepithelial fibroblasts. The propagating Ca^{2+} waves evoked by touch were also observed in subepithelial fibroblasts isolated from sl/sl-type rats (ET_B receptor gene mutant) and no significant differences between wild-type and sl/sl cells were observed (data not shown).

Cell contraction synchronized with propagating Ca^{2+} waves

When a subepithelial fibroblast was stimulated by touch, concomitantly with Ca^{2+} wave propagation, transient cellular

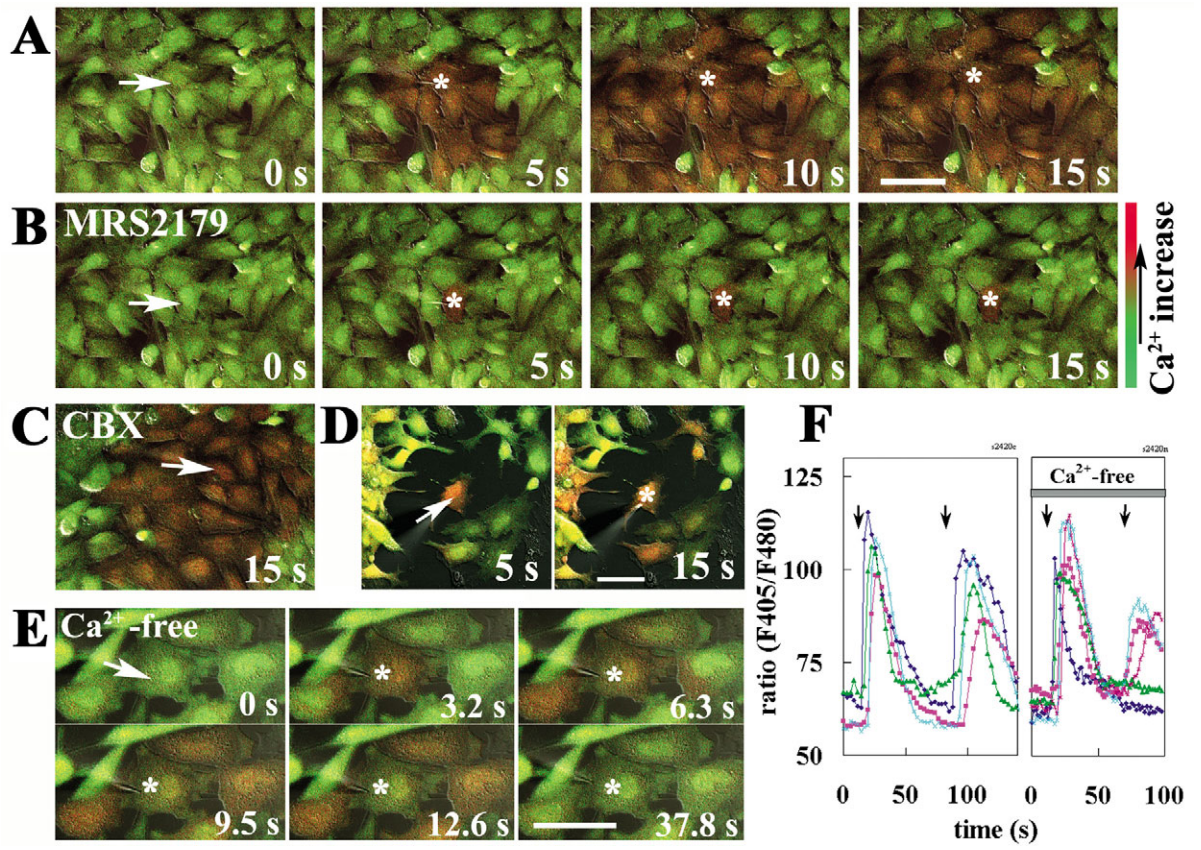

Fig. 4. Mechanical stimulation, 'touch', induces intercellular Ca^{2+} waves. Two images of indo-1 fluorescence (F405 and F480) were superimposed onto a Nomarski image where red represented F405 and green represented F480. So, cells in which Ca^{2+} increased turned red, as shown in color scale bar for Ca^{2+}-increases. Arrows and asterisks show touched cells. Magnification scales are 20 μm (A-C are the same magnification). (A) A Slight touch with a blunted thin glass rod induced intercellular Ca^{2+}-waves in subepithelial fibroblasts. See also supplementary material Movie 1. (B) MRS2179 (100 μM) inhibited touch-induced Ca^{2+} waves. The washing out of MRS2179 caused the recovery of Ca^{2+} waves. (C) CBX (100 μM), a blocker of gap junctions, is not effective on the initiation and propagation of Ca^{2+} waves. (D) Touch-induced Ca^{2+} waves could propagate to physically non-contacted cells. (E) Ca^{2+} waves were induced and propagated even in Ca^{2+}-free solution similarly under normal conditions. (F) Time courses of Ca^{2+} changes in individual cells are shown by increases in fluorescence ratios (F405/F480) after touch stimulation in normal Ringer (left) and in Ca^{2+}-free solutions (right). Cells touched for a second time also induced Ca^{2+} waves, although Ca^{2+} increases in touched cells (shown by dark-blue rhomboids) were suppressed in Ca^{2+}-free solution.

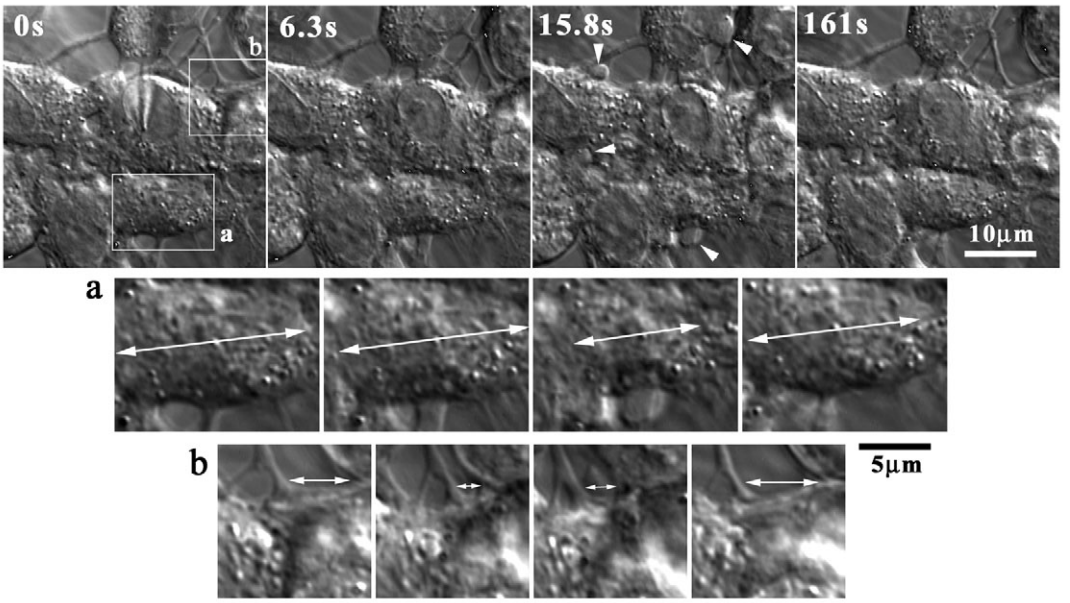

Fig. 5. Touch-induced cellular contractions propagate like a wave. Touching stimulations of a cell induced a propagation of contraction to the surrounding cells. The contraction seemed to spread with Ca^{2+} wave propagations. To show the contraction clearly, high magnification Nomarski images after stimulation were arranged with time. Arrowheads at 15.8 seconds indicate transient bleb formation in the cell membrane, sometimes accompanied with Ca^{2+} waves. Rows (a) and (b) show images magnified ×2 of two areas marked 'a' and 'b' in the top row (see image labeled 0 seconds). Arrows indicate corresponding regions to show the contractions. Supplementary material Movies 1, 2 facilitate the understanding of the contraction.

contractions were also propagated (Fig. 5, supplementary material Movies 1, 2). Cell contractions continued for a few tenths of a second and then relaxed. Sometimes, bleb formation in the cell membrane was observed in association with the contraction (Fig. 5 arrowheads, supplementary material Movies 1, 2). These transient and brief cellular contractions of subepithelial fibroblast networks may affect mechanical properties of villi.

Stretch-induced Ca^{2+} responses and ATP release

We applied another type of mechanical stimulation, a stretch, to subepithelial fibroblasts. The stretching (10-60%) of cells cultured on silicone elastomer induced Ca^{2+} increases under both conditions, i.e. in the presence (Fig. 6A) and the absence (Fig. 6B) of extracellular Ca^{2+}. The response was transient and disappeared after a few tens of seconds. The number of responsive cells increased dependent on an increase in stretch length, but not stretch duration or stretch speed (data not shown). In addition to the immediate Ca^{2+} response in the cells, propagating Ca^{2+} waves were observed, suggesting the release of ATP by stretch stimulation (supplementary material Movie 3).

Simultaneous to the Ca^{2+} measurement, we collected perfusates during the stretch at every minute. The ATP content in each fraction was measured with a luciferin-luciferase bioluminescence assay using a luminometer. The stretching of cells induced transient ATP release in a dose-dependent

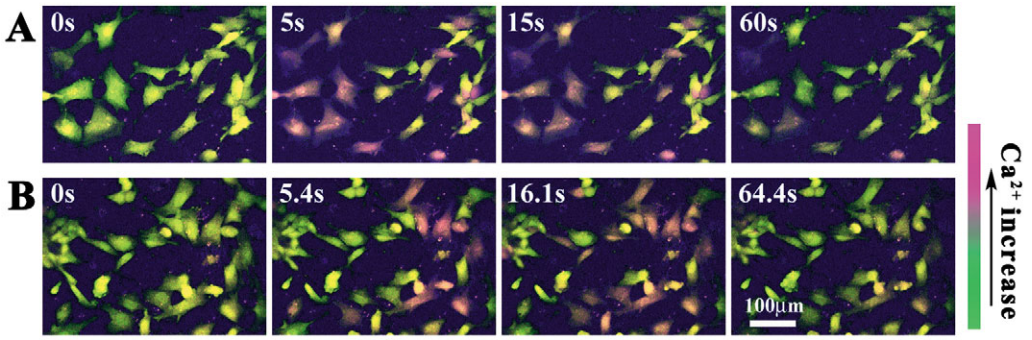

Fig. 6. Mechanical stimulation, 'stretch', induces Ca^{2+} responses and Ca^{2+} waves. Subepithelial fibroblasts were cultured on elastic chambers made by thin silicone elastomer membrane coated with collagen, and stretched using a stretch machine. Stretch stimulation (12% for 3 seconds) was applied to vertical direction in the figures between 0 seconds and 5 seconds. Ca^{2+} increases in the cells, measured by indo-1 fluorescence, occurred under the presence (A) and the absence (B) of extracellular Ca^{2+}. Wave-like Ca^{2+} responses were also observed in the process (see supplementary material Movie 3). Here, each image was a merged RGB color image of F405 (as red), F480 (as green) and F405/F480 (as blue). The scale for Ca^{2+} increase is shown in the right color bar.

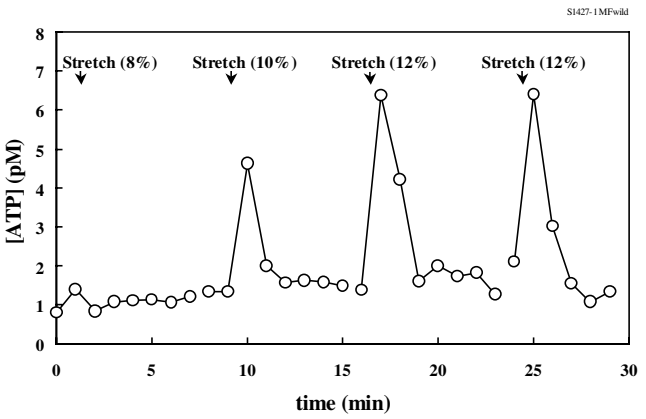

Fig. 7. Stretch induces ATP release. During the stretch stimulation, external solutions were perfused continuously at a speed of about 300 µl per minute and the perfusate was collected every minute. The ATP content in each elute was measured using a luciferin-luciferase bio-luminescence assay. ATP was released by stretching in a dose-dependent manner and repetitive stretching induced repetitive ATP release.

manner and ATP release was repeatedly induced (Fig. 7). In control cultures of flat shaped cells, ATP release was clearly observed by more than 10% stretching and increased with stretch length (Fig. 7). The amount of ATP released seemed to reach a plateau at about 40% stretching but stretching the cell more than about 60% induced large but non-reproducible ATP release (data not shown).

Suppression of mechano-sensitivities in stellate-shaped cells

Suppression of Ca^{2+} waves and Ca^{2+} responses

Subepithelial fibroblasts changed cell shape from flat to stellate with increasing intracellular cAMP levels. We checked the mechano-sensitivity in stellate-shaped cells that were incubated with DMEM/F12 containing 1 mM dBcAMP for 2 hours. Touch stimulation to a stellate-shaped cell did not evoke intercellular Ca^{2+} waves (Fig. 8A1), although a strong touch, which punctured the cell, induced a weak Ca^{2+} wave (Fig. 8A2). By the application of 1 nM ET1, the cell-shape changed to flat within 10 minutes. After that, touch to the flattened cell elicited Ca^{2+} waves similar to the controls (Fig. 8A3).

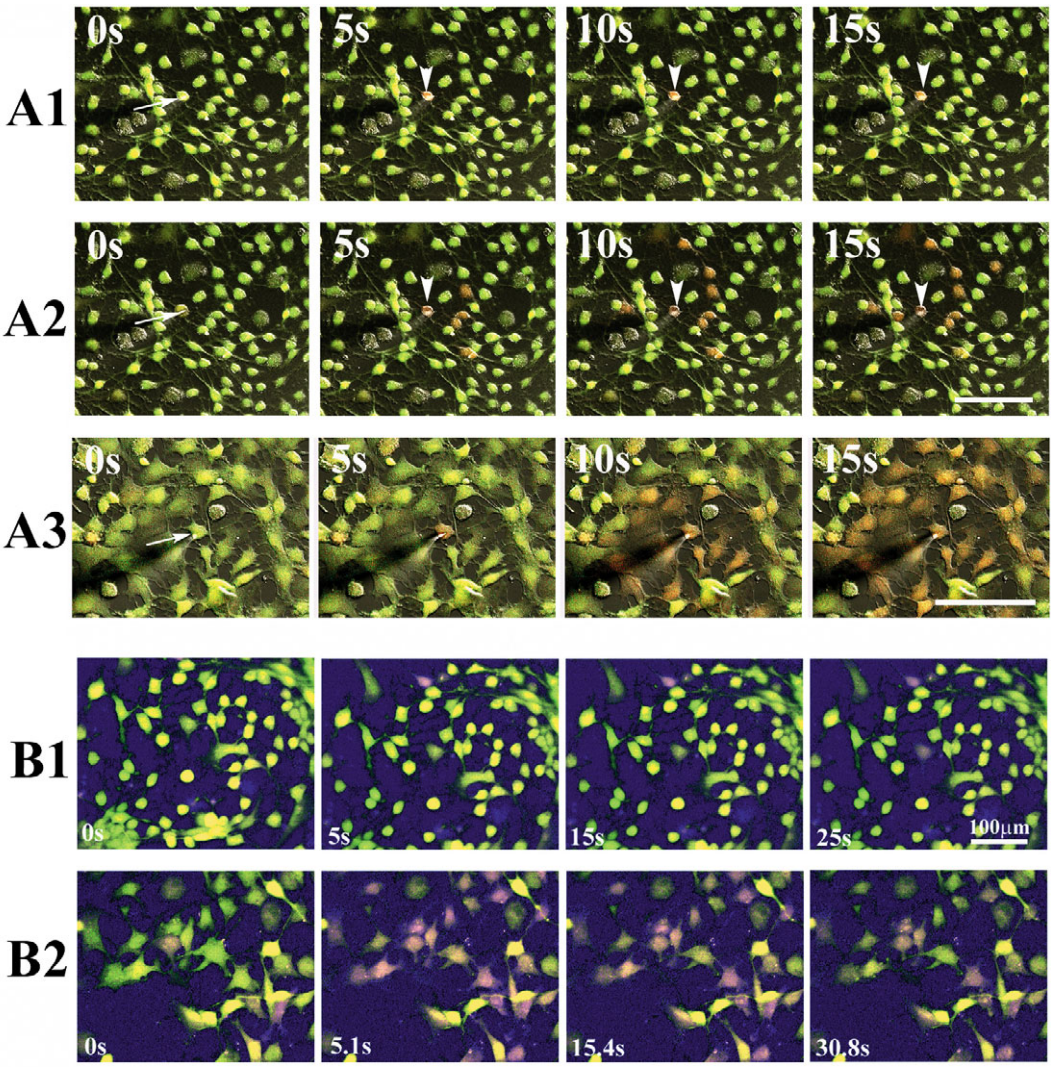

Fig. 8. Suppression of Ca^{2+} responses to touch and stretch in the stellate-shape cells. Stellate-shaped cells treated with 1 mM dBcAMP for 1.5 hours did not respond to touch (A1) and stretch (B1), although an extremely strong stimulation that punctured cells induced Ca^{2+} waves (A2). After 10 minutes treatment with 10 nM ET1, cells changed to flat, and sensitivity to touch and stretch was recovered (A3,B2). Arrows indicate touched cells. Ca^{2+} increases are shown by superimposed images: (A) F405 is red, F480 is green, Nomarski is gray; and (B) F405 is red, F480 is green, ratio of F405/F480 is blue. Similar results were obtained in over 10 dishes in several different cultures in both A and B. Bars, 100 µm.

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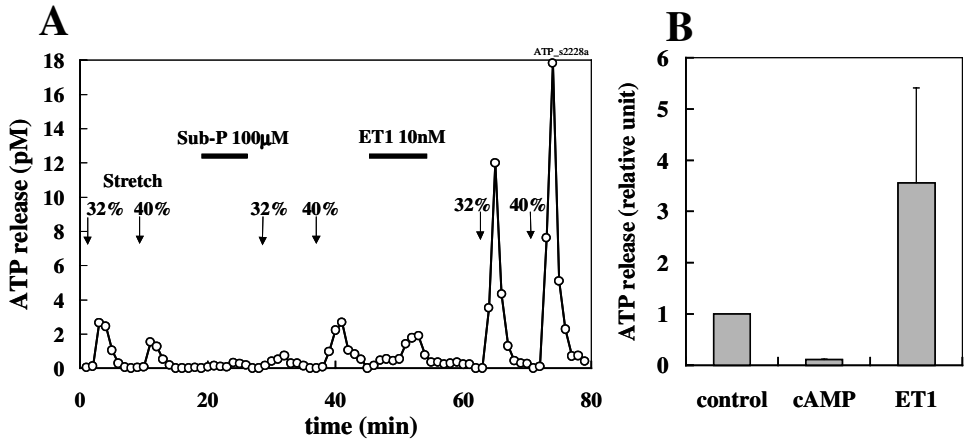

Fig. 9. Suppression of stretch-induced ATP-release in stellate-shaped cells. Subepithelial fibroblasts cultured on elastic chambers were treated with 1 mM dBcAMP for about 1 hour to induce the cells to change shape to stellate. During stretching, ATP contents in perfusates were measured every minute. (A) A typical example involving a strong stretch (32%, 40%) induces a small ATP release even in stellate-shaped cells. After ET1 (but not substance-P treatment) ATP release is obviously enhanced. (B) The average data for the effects of dBcAMP treatment and ET1 application on ATP release are shown, which were normalized by controls. *n*=5, bars show s.e.m.

Suppression of mechano-sensitivity in stellate-shaped cells also appeared during stretch responses. Stellate-shaped cells did not even respond to 40% stretching (Fig. 8B1). About 15 minutes after the application of 10 nM ET1, the cell shapes changed to flat and 32% stretching induced large Ca^{2+} responses (Fig. 8B2).

Suppression of ATP release

Suppression of Ca^{2+} waves in stellate-shaped cells suggests suppression in ATP release from touched cells and/or suppression of ATP sensitivity in surrounding cells. First we checked the effect on ATP release by stretch stimulation. In dBcAMP-treated stellate-shaped cells, stretching less than 24% did not induce any detectable ATP release (data not shown), but strong stretches (32-40%) induced a small release of ATP (Fig. 9A). Substance-P (Sub-P, 100 μM), which is known to induce Ca^{2+} increases but not morphological changes in subepithelial fibroblasts (Furuya et al., 1994), did not induce changes in ATP release (Fig. 9A). Conversely, ET1 could induce Ca^{2+} increases and cell shape conversion from stellate to flat (Furuya and Furuya, 1993; Furuya et al., 1994). Application of 10 nM ET1 induced slight ATP release and 15 minutes after ET1 treatment, stretch-induced ATP releases increased more than four times (Fig. 9A). On average, dBcAMP treatment (over 1 hour) suppressed ATP release by stretch (12-40%) to about 11% of that under control conditions and further treatment with ET1 (10 nM, 10-20 minutes) recovered or enhanced ATP release about 3.5 times (Fig. 9B).

Suppression of ATP sensitivity

Secondly, we measured the effect of cell shape on ATP sensitivity (Fig. 10). The Ca^{2+} response to ATP (10 μM) was suppressed to about 40% in dBcAMP treated stellate-shaped cells. Suppression was recovered after flat conversion by 10 nM ET1 treatment. Even in stellate-shaped cells, 100 μM ATP induced full responses nearly the same as in the flat-shaped cells (data not shown).

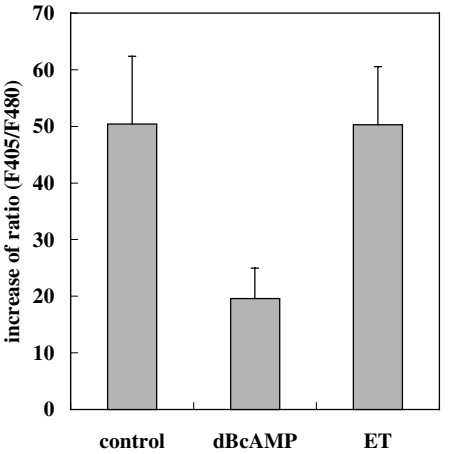

Fig. 10. Suppression of ATP sensitivity in stellate-shaped cells. Ca^{2+} responses to 10 μM ATP were measured in control (flat-shape), 1 mM dBcAMP-treated stellate-shaped cells and ET1-treated flat-shaped cells. Average data and s.e.m. (bars) were obtained from more than 70 cells in three or four experiments.

Propagation of Ca^{2+} waves from subepithelial fibroblasts to neural cells (NG108-15)

The uptake of food and water evokes reflex in the villi. It is thought that sensory cells in the villi respond to serotonin and ATP, which are secreted from enterochromaffin cells in epithelial layer, and elicited reflex (Buchan, 1999; Höfer et al., 1999; Cooke et al., 2003). In the villi, however, nerve terminals are closely associated to the network of subepithelial fibroblasts and not to enterochromaffin cells. From their location and our findings that subepithelial fibroblasts are mechano-sensitive, we postulate that subepithelial fibroblasts work as the mechano-sensor in the villi. To confirm this idea we examined signal propagation from subepithelial fibroblasts to neurons using a co-culture of subepithelial fibroblasts with neural cells (NG108-15 cells). Neuroblastoma × glioma

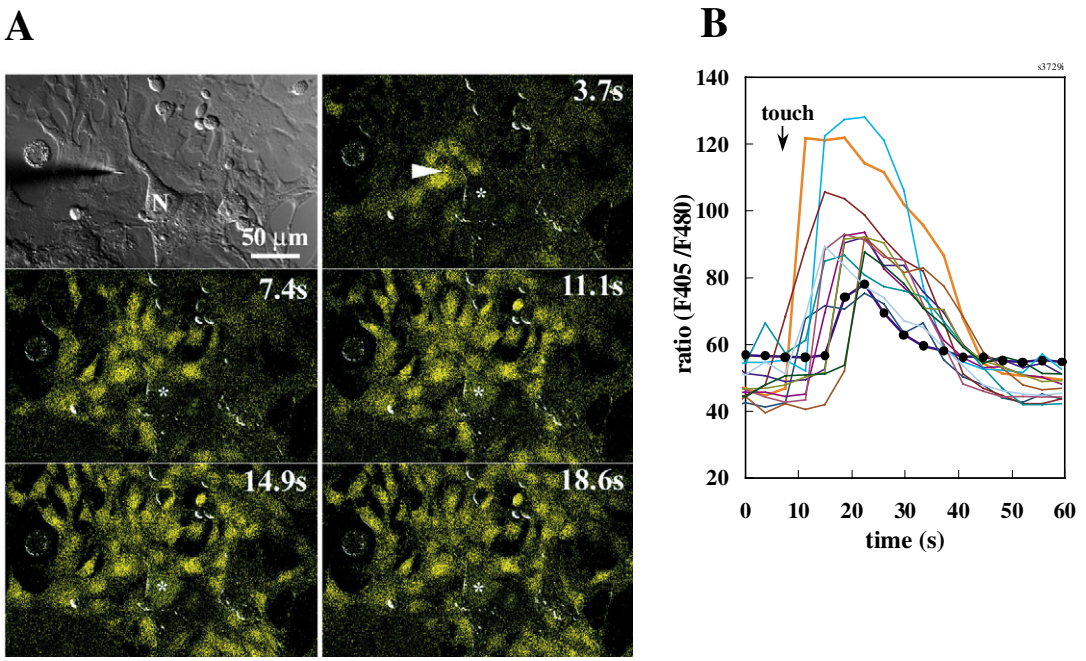

Fig. 11. Propagation of Ca^{2+} waves from subepithelial fibroblasts to neuronal cells. (A) Differentiated NG108-15 cells were seeded onto the culture of subepithelial fibroblasts and co-cultured for 1 day (upper left, Nomarski image). A NG108-15 cell is indicated by 'N' in the figure. Ca^{2+} waves were generated by touching a subepithelial fibroblast (3.7-18.6 seconds). Each image is superimposed onto the ratio (F405:F480) image (yellow) with Nomarski images. Yellow parts indicate the cells in which Ca^{2+} increased. Co-cultured NG108-15 cell (indicated by an asterisk) was activated with the Ca^{2+} waves. (B) Time courses of Ca^{2+} wave propagation in subepithelial fibroblasts (colored lines) and Ca^{2+} increases in NG108-15 cells (black line with filled circles) are shown by the ratio (F405/F480) in each cell.

hybrid NG108-15 cells were differentiated with 1 mM dBcAMP for 5-7 days and they exhibited neuronal characteristics in ultrastructural aspects and electrophysiological properties (Nelson et al., 1976; Hamprecht, 1977; Furuya and Furuya, 1983). Differentiated NG108-15 cells were rich in P2Y1, P2Y2, P2Y4 and P2X4 mRNA by RT-PCR analysis (data not shown). By touching a subepithelial fibroblast with a thin glass pipette, Ca^{2+} waves were propagated via the network of subepithelial fibroblasts. During the Ca^{2+} wave propagation, NG108-15 cells were activated by the Ca^{2+} wave, which induced intracellular Ca^{2+} increase (Fig. 11A,B). This finding indicates the possibility that ATP released by mechanical stimulation of the network of subepithelial fibroblasts can activate sensory neurons in the intestinal villi.

Discussion

Physiological roles of the network of subepithelial fibroblasts

From the esophagus to the anus, subepithelial fibroblasts form a cellular network under the absorptive epithelium and separate the epithelium from the lamina propria where blood vessels, smooth muscles and neurons are located. From various reports and our data, several functions of subepithelial fibroblasts are considered as shown in Fig. 12.

(1) Subepithelial fibroblasts secrete extracellular matrices and form basal lamina, which are perforated by numerous fenestrations (Komuro, 1985; Komuro and Hashimoto, 1990; Toyoda et al., 1997). Under basal lamella, dense collagenous fibrils and subepithelial fibroblasts form subepithelial reticular sheets. Basal lamina and subepithelial reticular sheets work together as a sieve for various substances and immune cells. In addition to their direct sieve function, they may control the permeability of epithelium to ions, nutrients and water by releasing various cytokines, such as TGF-β, TNF-α, HGF, PGEs. These cytokines are secreted from subepithelial fibroblasts (Valentich et al., 1997; Plateroti et al., 1998) and modify the assembly of tight junctions of epithelium to change their permeability (Beltinger et al., 1999; Walsh et al., 2000). So, the subepithelial fibroblast network may work as a barrier or sieve by itself and by controlling the epithelium in the intestine. Subepithelial fibroblasts change their shape quickly and dramatically by ETs applications depending on intracellular cAMP levels (Furuya and Furuya, 1993), suggesting that the barrier or sieve properties are controlled locally and dynamically in the villi.

(2) Subepithelial fibroblasts are rich in α-smooth muscle actin and their cellular networks ensheath the laminar propria of intestinal villi as a contractile network. So, they may work as a mechanical frame to keep the structure of the villi (Fig. 12). We think the structure and mechanical properties of villi are kept by not only smooth muscle in laminar propria but also subepithelial fibroblast networks. Furthermore, this mechanical frame is not static and the properties may be regulated by the morphological changes of subepithelial fibroblasts. In this paper, we showed that subepithelial fibroblasts made transient (10-20 s) contractions synchronizing to Ca^{2+} wave propagations (Fig. 5). The contractions were due to the released ATP. ETs also induced brief contractions of subepithelial fibroblasts, although the duration was rather

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longer and sometimes oscillatory [data not shown, but Ca^{2+} changes were shown elsewhere (Furuya et al., 1994)]. The dBcAMP-treated stellate shaped cells did not show such contraction. So, the mechanical properties of villi may change locally and dynamically by changing cell shape and contracting the network.

In addition to these barrier/sieve and frame functions, here, we propose that (3) subepithelial fibroblasts work as a mechano-sensor in the intestine (Fig. 12). This proposal is based on our findings that subepithelial fibroblasts are extremely sensitive to mechanical stress, which induces ATP release, and that the sensitivity changes drastically with cell shape.

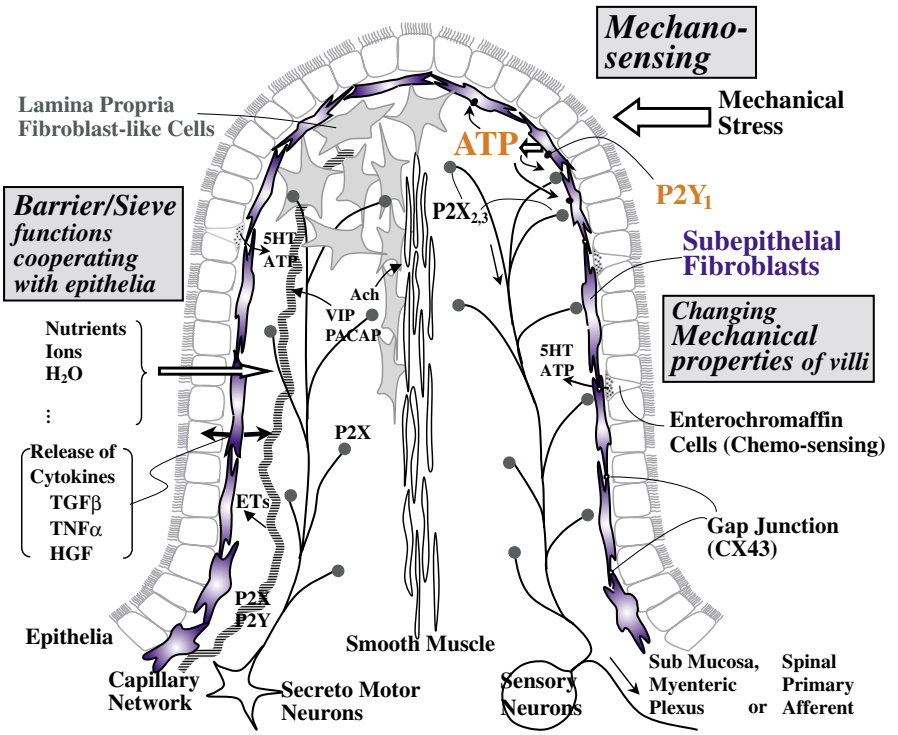

Fig. 12. A model for subepithelial fibroblasts working as a mechano-sensor and other functions in the intestinal villi. Food and water intake deform villi and cause mechanical stress to the network of subepithelial fibroblasts (dark-blue cells). ATP is released from subepithelial fibroblasts by the mechanical stimulation and activates P2Y1 on surrounding cells as an autocrine mediator. The released ATP also activates the nerve endings of mucosal sensory neurons via P2X receptors. This represents a mechanosensing process in the villi and may contribute to the peristaltic reflex. Subepithelial fibroblast networks communicate with α-SMA-negative lamina propria fibroblasts-like cells (gray cells) and they form three-dimensional networks. They closely contact capillary networks, sensory and motor neuronal networks, smooth muscles, and epithelium in the villi. Subepithelial fibroblasts communicate among these cell systems via released ATP and other humoral factors, such as TGF-β, TNF-α. From these views, we consider subepithelial fibroblasts work as barrier or sieve, mechano-sensor, mechanical frame, and signal transduction machinery in the villi. These functions are most likely locally and dynamically regulated in the villi by the rapid changes in cell-shape and mechano-sensitivity.

Mechano-sensitive network through ATP release

Mechanical stimulations, 'touch' and 'stretch', to subepithelial fibroblasts induce an increase in intracellular Ca^{2+} (Fig. 4; Fig. 6). The Ca^{2+} responses to mechanical stimuli are mainly due to the release from intracellular stores because they occur even in Ca^{2+}-free solution (Fig. 4; Fig. 6). Although only partial, there is a contribution from the influx of extracellular Ca^{2+} (Fig. 4F).

Mechanical stimulations also induce the release of ATP, as clearly shown in the dose-dependent response to stretching, where ATP contents in perfusates were measured using luciferine-luciferase bioluminescence assays (Fig. 7). The released ATP conveys intercellular Ca^{2+} waves (Fig. 4) or wave like responses (Fig. 6) by activating P2Y1 in surrounding cells.

Ca^{2+} waves were reported to propagate via IP3 transfer through gap junctions in some cells (Charles et al., 1992). However, this is not the case, because Ca^{2+} waves propagated between colonies separated by space (Fig. 4B), and were suppressed by the treatment with P2Y antagonists MRS2179 (Fig. 4B) and suramin, but not suppressed by the treatment with gap junction inhibitor, CBX (Fig. 4C). In addition, as discussed in later, in dBcAMP treated stellate-shaped cells, touch-induced Ca^{2+} waves were suppressed, although gap junction permeability was still open (Furuya et al., 2005). This also confirmed that mechanically induced Ca^{2+} waves were propagated via released ATP but not via gap junctions.

P2Y1 as a functional ATP receptor in subepithelial fibroblasts

To determine subtypes of ATP receptors in subepithelial fibroblasts, immunohistochemical (Fig. 1), RT-PCR (Fig. 2A), western blotting (Fig. 2C), and physiological and pharmacological (Fig. 3) analyses were done. By the Ca^{2+} measurements, the responses in Ca^{2+}-free medium did not differ from the responses in normal Ringer solution for the first time, indicating P2Y contribution to the responses (Fig. 3B). We deduced the following potency hierachy for the nucleotides we used: 2MeSATP>ADP⩾ATP>>UTP>UDP, which clearly suggests functional subtype being P2Y1. This result was supported by the findings that P2Y1 antagonist, MRS2179, blocked Ca^{2+} responses to 2MeSATP completely but P2X1 antagonist, MRS2159, did not (Fig. 3D).

By the RT-PCR measurements, in addition to P2Y1 mRNA, P2Y2, P2Y4 and P2X1 mRNA are also expressed in primary cultures (Fig. 2A). However, UTP and UDP, which are potent agonists of P2Y4 and P2Y2, were not very effective in Ca^{2+} responses (Fig. 3A), and only P2Y1 immunoreactivity was observed (Fig. 2) but not P2Y2 and P2Y4 (data not shown) in subepithelial fibroblasts of rat small intestine and colon. Human colonic fibroblasts 18Co cells, which exhibit similar characteristics to subepithelial fibroblasts (Valentich et al.,

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1997), predominantly express P2Y1 mRNA (Fig. 2B) and P2Y1 protein (Fig. 2C). In rat duodenum, P2X1 localizes within the capillary network of lamina propria, and P2X2 in the intrinsic sensory neurons (Gröschel-Stewart et al., 1999; Castelucci et al., 2002). So, the presence of small amounts of P2Y4, P2Y2 and P2X1 mRNA in the primary culture of subepithelial fibroblasts may originate from a contamination of other cell types in the lamina propria such as capillary endothelial cells, neurons, and smooth muscle cells etc. From these data, we concluded that P2Y1 is predominantly expressed and functional ATP receptor in subepithelial fibroblasts.

Changes in mechano-sensitivity with cell shape

In addition to the finding that subepithelial fibroblasts are highly mechano-sensitive, we demonstrated that mechano-sensitivities change drastically with cell shape. Subepithelial fibroblasts change morphology to stellate-shape by dBcAMP or forskolin treatment (intracellular cAMP raising reagents), and change to flat-shape by ET1 or ET3 treatment (Furuya and Furuya, 1993). Morphological changes were reversible and fast, e.g. the stellate to flat shape conversion by ETs occurred within 10 minutes (Furuya and Furuya, 1993). In dBcAMP-treated stellate-shaped cells, mechano-sensitivities were suppressed, i.e. touch-induced Ca^{2+}-waves (Fig. 8A), stretch-induced Ca^{2+} increase (Fig. 8B) and stretch-induced ATP-release (Fig. 9) were abolished.

The disappearance of Ca^{2+}-waves in mechanical stimulation is mainly due to the suppression of ATP release in dBcAMP-treated stellate-shaped cells. However, there was a contribution from the suppression of ATP sensitivity in stellate-shaped cells (Fig. 10). The suppression of ATP sensitivity might be due to an endocytosis of receptors or an uncoupling of receptors and Ca^{2+} signaling pathways, because suppression was quick and there were no detectable differences between control flat- and dBcAMP treated stellate-shaped cells in the expression of P2Y1 mRNA and protein (data not shown). Alternatively, a contribution of other pathway, such as cADP-ribose/Ca^{2+} signaling system that enhanced the Ca^{2+} increase upon P2Y stimulation in 3T3 fibroblasts (Bruzzone et al., 2003), is also applicable.

Suppression of mechano-sensitivity in stellate-shaped cells disappears when cells are flattened by ETs (10 nM) treatment (Figs 8, 9). Sometimes the mechano-sensitivity after ETs treatment was enhanced more than the control. Recovery of mechano-sensitivity did not occur upon the addition of substance-P, which induces Ca^{2+} increases but not morphological changes, suggesting a necessity for morphological changes in subepithelial fibroblasts. The cell morphology is simply controlled by intracellular cAMP level in subepithelial fibroblasts. So, mechano- and ATP sensitivities may be regulated by intracellular cAMP-mediated signal transduction. To investigate the mechanism of mechano-sensing, it would be important to clarify which is primary for mechano-sensitivity, cell morphology or cAMP-mediated signaling.

Endothelins and endothelin-converting enzymes localize in blood vessels, cryptic epithelial cells, mast cells and macrophages in the gastrointestinal tract (Liu et al., 1998; Egidy et al., 2000). The network of subepithelial fibroblasts is adjacent to these cells in vivo. So, subepithelial fibroblasts could change cell-shape by these ETs and the mechano-sensitivity of the network may be regulated dynamically and locally in the villi.

Subepithelial fibroblasts communicate mutually not only with humoral factors but also via gap junctions. Interestingly, unlike the mechano-sensitivity, gap junction permeability did not change with cell shape, and usually stayed open (Furuya et al., 2005). So, subepithelial fibroblast networks always form a syncytium in villi.

Mechanism of ATP-release

In many types of cells and tissues, mechanically induced ATP-release and following activation of ATP receptors are observed, and ATP release is thought to be an important and ubiquitous mechano-sensing machinery (Schwiebert, 2000; Burnstock, 2001a; Arcuino et al., 2002; Furuya et al., 2004). ATP is released by several pathways: the exocytosis of vesicles (Osipchuk and Cahalan, 1992; Bodin and Burnstock, 2001a; Coco et al., 2003); anion channels (Sabirov et al., 2001; Hisadome et al., 2002); hemi-channels of gap junctions (Cotrina et al., 1998a; Stout et al., 2002); transporters (Roman et al., 1997; Bodin and Burnstock, 2001b); and transient nonselective membrane channels (Arcuino et al., 2002). However, the details of the mechanism, the molecule, and an overview of ATP release have not yet been clarified.

In subepithelial fibroblasts treated with dBcAMP or forskolin, thick stress fibers depolymerize during cell shape changes from flat to stellate, whereas in subepithelial fibroblasts treated with ETs the stress fibers re-polymerize and stellate-shaped cells changed to flat (Furuya and Furuya, 1993). According to these morphological changes, the sensitivity of ATP-release to mechanical stimulation is remarkably changed. By treatment with Y27632, a Rho kinase inhibitor, stress fibers in flat shape cells are diminished and mechanically induced ATP-release is inhibited, although ATP sensitivity is not changed (our preliminary results). These findings suggest a contribution from the actin cytoskeleton on ATP-release in subepithelial fibroblasts as indicated in cultured astrocytes (Cotrina et al., 1998b). The mechanism of the regulation is now under investigation.

The mechano-sensor in the intestine

Food uptake and digestion may give rise to chemical and mechanical signals that induce a peristaltic reflex in the gut. The signals control motility, secretion and immune defenses in local and/or through central, and neural and/or non-neural pathways (Buchan, 1999; Furness et al., 1999; Furness et al., 2004). This means that the intestine is regarded as a sensory organ (Furness et al., 1999). Even in isolated gut, distension caused by intraluminal hydrostatic pressure induces peristalsis and there seems to exist several mechano-sensors in mucosa, submucosa and muscle layer including myenteric plexus (Tsuji et al., 1992). In the mucosa, it was suggested that luminal stimuli release sensory mediators from mucosal epithelium, which then activate nerve terminals of sensory neurons (Buchan, 1999; Höfer et al., 1999; Burnstock, 2001a; Cooke et al., 2003). Cholecystokinin (CCK) and serotonin are reported to be chemo-mediators that excite nerve terminals in mucosal

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sensory neurons, and are released by luminal stimuli (Kirchgessner et al., 1992; Wade et al., 1996; Eastwood et al., 1998). ATP is also reported to activate nerve terminals of intrinsic sensory neurons via P2X2 and/or P2X3 (Bertrand and Bornstein, 2002; Castelucci et al., 2002; Poole et al., 2002; Bian et al., 2003; Ren et al., 2003), or P2Y1 (Cooke et al., 2004). In P2X2 or P2X3 gene deleted mice, the intraluminal pressure-induced peristalsis was inhibited (Bian et al., 2003; Ren et al., 2003). In mucosa, ATP is thought to be released from enterochromaffin and from epithelial cells by mechanical stress of the intestine (Burnstock, 2001a; Cooke et al., 2003).

However, there is a considerable distance from epithelial to neuronal termini. Between them, there exist basal lamina and also a network of subepithelial fibroblasts that are sensitive to serotonin and ATP (Furuya et al., 1994). Furthermore, subepithelial fibroblasts are sensitive to mechanical stress and release ATP as shown herein. From their location and from their displayed characteristics, subepithelial fibroblasts are thought to plausibly be the mechano-sensor in the intestinal villi. Actually, nerve terminals are located near subepithelial fibroblasts in the villi (Güldner et al., 1972; Desaki et al., 1984). The finding that neural cells (NG108-15 cells) co-cultured on subepithelial fibroblasts could be activated by mechanically induced Ca^{2+} waves in subepithelial fibroblasts supports the idea (Fig. 11). So, we propose that subepithelial fibroblasts release ATP upon stretch or distension of villi and that the ATP acts on P2X2 and/or P2X3 receptors on intrinsic sensory enteric neurons to regulate peristalsis and on extrinsic sensory neurons originating in dorsal root ganglion to induce nociception.

In the urinary bladder, a similar mechanism is working for nociception of bladder fullness, although the mechano-sensing or ATP releasing cells are thought to be epithelial (urothelial) cells (Cockayne et al., 2000; Vlaskovska et al., 2001). Recently, however, suburothelial myofibroblasts have been recognised as an additional regulator of mechano-signaling. Suburothelial myofibroblastslocate are located under the bladder epithelium, form networks as a syncytium via gap junctions, and have intermediate filament vimentin and P2Y receptors (Sui et al., 2002; Sui et al., 2004; Wiseman et al., 2003; Wu et al., 2004). These properties are similar to the subepithelial fibroblasts in the intestine, although their mechano-sensitivity has not been described yet.

Other signaling functions in the intestine

Axons, which contain synaptic vesicles and/or large granular vesicles, are located close to the cell bodies and cell processes of subepithelial fibroblasts (Desaki et al., 1984). This means that nerve terminals of motor neurons [cholinergic neurons, non-cholinergic vasoactive intestinal peptide (VIP) neurons, and so on] are also in close contact with subepithelial fibroblasts. Although subepithelial fibroblasts in primary culture do not respond to acetylcholine, VIP (Furuya et al., 1994) and carbachol (Fig. 3C) according to Ca^{2+} measurements, 18Co cells secrete prostaglandin E2 by carbachol stimulation (Valentich et al., 1997). It is possible that ATP and transmitters released from motor neurons may affect subepithelial fibroblasts, directly and indirectly.

The subepithelial fibroblast network also overlays the capillary network in the villi. Ca^{2+} waves evoked by mechanical stimulation may propagate to capillaries, and regulate absorption of nutrients or regulate blood flow by cell contraction. Moreover, subepithelial fibroblasts represent cellular communication with α-SMA-negative fibroblast-like cells, which enclose smooth muscles in the lamina propria (Güldner et al., 1972; Toyoda et al., 1997; Adegboyega et al., 2002). Although these fibroblast-like cells do not synthesize collagen fibrils, they are rich in ET receptors as subepithelial fibroblasts (Furuya et al., 1990). So, it is possible that signals evoked by mechanical stimulation in subepithelial fibroblasts propagate not only to sensory neurons, but also to blood capillaries, and also to smooth muscles via fibroblast-like cell processes.

The characteristics of subepithelial fibroblasts are very similar to those of astrocytes, i.e. they possess various types of receptors for neuro- and vaso-active substances, undergo rapid and reversible morphological changes between flat and stellate depending on intracellular cAMP levels (Goldman and Abramson, 1990), and induce the cell shape-dependent propagation of Ca^{2+} waves (Cotrina et al., 1998b). Both cells form a syncytium via gap junctions, and their networks are in close contact with neuronal and capillary networks. Upon induction by mechanical stress, both subepithelial fibroblasts and astrocytes release ATP, and Ca^{2+} waves propagate not only within the cellular network, but also propagate to adjacent neurons and capillaries (Fields and Stevens, 2000; Mulligan and MacVicar, 2004). However, there are several differences between subepithelial fibroblasts and astrocytes. Subepithelial fibroblasts are rich in ET_A receptors and P2Y1 receptors, and gap junction permeability is regulated independent of intracellular cAMP level. Astrocytes express more dominantly ET_B receptors and P2Y2 receptors than ET_A receptors and P2Y1 receptors (Hori et al., 1992; Sasaki et al., 1998; Zhu and Kimelberg, 2001), and gap junction permeability is regulated in a cAMP-dependent manner (Giaume et al., 1991; Giaume and McCarthy, 1996; Blomstrand et al., 2004). Subepithelial fibroblasts and astrocytes are believed to play a key role in cell-cell communication in the gastrointestinal tract, and central and peripheral nervous system, respectively.

In summary, the subepithelial fibroblast network forms a syncytium under the epithelium in the villi, responds to many kinds of baso- and neuro-active substances, and changes shape rapidly or contracts transiently. Subepithelial fibroblasts are also highly sensitive to mechanical stress and release ATP. Subepithelial fibroblast networks are not only connected with lamina propria fibroblast-like cells, but also closely contact the capillary network, sensory and motor neuronal networks, smooth muscles, and epithelium in the villi. Subepithelial fibroblasts communicate among these cell systems via ATP release and other humoral factors, such as TGF-β, TNF-α. In the light of these findings, we conclude that subepithelial fibroblasts work as: (1) a barrier or sieve; (2) a mechanical frame; (3) a mechano-sensor; and (4) signal transduction machinery in the villi. These functions are likely regulated locally and dynamically in the villi by rapid cell-shape changes and cell-shape dependent mechano-sensitivities, which may play crucial roles in intestinal functions.

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