DIFFERENTIAL CELLULAR EXPRESSION OF THREE TYPES OF INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR IN RAT GASTROINTESTINAL EPITHELIUM

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ABSTRACT

In epithelial tissues of rat gastrointestinal tract, inositol 1,4,5-trisphosphate receptor type-1, type-2 and type-3 were immunolocalized with type-specific monoclonal antibodies (KM1112, KM1083 and KM1082, respectively). The immunoreactivities for inositol 1,4,5-trisphosphate receptor type-1 and type-3 were localized predominantly in gastric parietal cells, whereas no immunoreactivity was observed in gastric chief cells. Inositol 1,4,5-trisphosphate receptor type-2 was immunolocalized exclusively in goblet cells of small intestine, especially of duodenum, but not in goblet cells of colon. Western blotting analysis verified the immunolocalizations of the inositol 1,4,5-trisphosphate receptor types in these tissues. The differential expression of the inositol 1,4,5-trisphosphate receptor types may be responsible for various gut secretory functions.

The intracellular second messenger inositol 1,4,5-trisphosphate (IP₃), which is produced through phosphatidyl-inositide (PI) turnover in response to extracellular stimuli, mediates the release of calcium (Ca²⁺) from intracellular stores upon binding to its specific receptor (1). IP₃ receptor (IP₃R) type-1, type-2 and type-3 (IP₃R-1, IP₃R-2 and IP₃R-3, respectively) have been molecularly cloned (2, 8, 13-15, 20, 21, 23, 24), and the amino acid sequences of the three types display strong homology in membrane-spanning and IP₃-binding domains (24). Among various epithelial tissues of mammals, the IP₃R types have been differentially localized (4). Physiological and pharmacological findings suggest that specific forms of the IP₃R probably play critical roles for specific epithelial cell functions.

The mucosal epithelium of gastrointestinal tract has two main functions; the secretion of mucus and

absorption of food. Recent studies with various extracellular stimuli, such as neurotensin (3), 1,25-(OH)₂-D₃ (10) and carbachol (5) have demonstrated that Ca²⁺ mobilization coupled with IP₃ production takes place in gastrointestinal epithelial cells. From electrophysiological studies using intestinal epithelial-derived cell lines, it was suggested that intracellular Ca²⁺ mobilization potentiates Cl⁻ secretion (5,17). It is interesting to know which types of IP₃R are present in specific cell types of the gastrointestinal epithelium. Reverse transcription-polymerase chain reaction (RT-PCR) and *in situ* hybridization analyses have detected the expression of the IP₃R-1 and IP₃R-3 mRNA in adult gastrointestinal tissues (2, 6, 20). Maranto (13) immunologically observed the expression of the IP₃R-3 in rat jejunal crypt and villus epithelial cells using affinity-purified antibodies against the C-terminal portion of IP₃R-3. However, little is known about cellular localizations of the IP₃R-1, -2 and -3 in gastrointestinal epithelial tissues.

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Fig. 1 Immunocytochemical localizations of IP₃R-1 and IP₃R-3 in epithelium of rat stomach. Tissue sections (10 μ m thick) were incubated with mAb KM1112 (A and C) and mAb KM1082 (B and D) or mAbs KM1112 and KM1082 pre-incubated with the immunogen peptides (E and F, respectively). The sections were counterstained with Mayer's hematoxylin and 0.5% eosin Y. Scale marker, 25 μ m

We have previously prepared three monoclonal antibodies (mAbs); each of the mAbs is type-specific (22). In the present study, we investigated the distributions of the immunoreactivities for IP₃R-1, -2 and -3 in rat gastrointestinal epithelial tissues using the type-specific mAbs. Their characteristic expression patterns will be discussed in terms of gastrointestinal functions.

MATERIALS AND METHODS

Animals and Monoclonal Antibodies

Male rats (Wistar, 8–10 weeks old) were obtained

from Nippon SLC (Shizuoka, Japan). Mouse monoclonal antibodies KM1112 (IgG1), KM1083 (IgG2a) and KM1082 (IgG1) that specifically recognize 15 amino acids of the COOH-terminus of human IP₃R-1, IP₃R-2 and IP₃R-3, respectively, were prepared as described (22).

Preparation of Membrane Fraction

After animals were anesthetized with pentobarbital sodium, the gastrointestinal tissues were rapidly dissected and frozen. The tissues were pulverized in liquid nitrogen, and then homogenized in 5

Fig. 2 Immunocytochemical localization of IP₃R-1 in epithelium of mouse stomach. Tissue sections (10 μ m thick) were incubated with mAb 18A10 (A) and mAb 10A6 (B). The locations of their epitopes are indicated in references 10 and 11. The sections were counterstained with Mayer's hematoxylin. Scale marker, 25 μ m

vol of ice-cold 10 mM Tris-HCl (pH 8.0 at 0°C) containing 0.32 M sucrose, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μ M pepstatin A, 10 μ M leupeptin and 1 mM 2-mercaptoethanol. The homogenates were centrifuged at $2,000 \times g$ for 5 min at 4°C. The supernatants were further centrifuged at $90,000 \times g$ for 20 min at 2°C. The pellets were resuspended in 50 mM of Tris-HCl (pH 8.0 at 0°C) containing 1 mM EDTA and 1 mM 2-mercaptoethanol. These membrane suspensions were subjected to Western blotting.

Western Blotting Analysis

The membrane fraction dissolved in the sample buffer (final concentrations; 4% SDS, 10% β -mercaptoethanol, 125 mM Tris-HCl, pH 6.8 and 20% glycerol) was subjected to 5% SDS-PAGE, followed by electroblotting onto nitrocellulose membranes (HybondTM-ECL, Amersham, U.K.). The blots were blocked with skim milk and immunoreacted with the mAbs KM1112, KM1083 or KM1082, and then with horseradish peroxidase (HRP)-conjugated anti-mouse IgG (Amersham). As a pre-absorption control, the mAbs were pre-incubated with 2 μ g/ml of their immunogen peptides prior to analysis.

Immunohistochemistry

Wistar rats were intracardially perfused with phos-

phate-buffered saline (PBS) followed by freshly prepared 2% paraformaldehyde fixative. Tissues were dissected and postfixed with the same fixative at 4°C for 2–4 h, and then infiltrated with 20% sucrose. Cryosections (10 μ m thick) were incubated with normal horse serum (1:50 dilution, Vector) in PBS for 30 min, and further incubated with the mAbs against IP₃R proteins at 4°C overnight. The sections were sequentially reacted with anti-mouse IgG avidin-biotinylated HRP complex (ABC) (Vector). The immunoreactivities were visualized with 3,3'-diaminobenzidine. The sections were counterstained with Mayer's hematoxylin and 0.5% eosin Y, or alcian blue (pH 2.5). The antigen pre-absorption control gave the same results as the primary antibody omitted.

RESULTS

The expression of mRNAs for type-1 and type-3 IP₃Rs in gastrointestinal tissues has been shown by RT-PCR, Northern blot, and *in situ* hybridization analyses (2, 6, 13, 20). However, these studies could not elucidate the cell types responsible for the expression. Furthermore, there have been no data as to the expression of IP₃R-2 in epithelium of gastrointestinal tissues. To define the cellular localization of each of the three IP₃R types in rat gastrointestinal epithelium, we performed immunocytochemical studies using the type-specific mAbs KM1112, KM1083 and KM1082.

IP₃R-1 and IP₃R-3 Localized in Parietal Cells of Stomach

Immunosignals of mAbs KM1112 and KM1082 were detected in gastric mucosal cells of cardia, body and pylorus of rat stomach (Fig. 1, A and B). These immunoreactive cells proved to be gastric parietal cells, since they were counterstained by 0.5% eosin Y. On the other hand, chief cells, which are not eosinophilic, did not exhibit any immunosignals. Fig. 1, C and D show the colocalization of the immunoreactive and eosinophilic regions in gastric parietal cells. The immunoreactivities were completely abolished by pre-incubation with the immunogen peptides (Fig. 1, E and F). These results indicate the localizations of IP₃R-1 and IP₃R-3 in gastric parietal cells. The localization of the IP₃R-1 in this cell-type was also verified by immunohistochemistry of mouse stomach with three rat mAbs against the mouse IP₃R-1: mAb 4C11, mAb 10A6, and mAb 18A10 (11, 12). Each of the mAbs gave immunosignals identical to Fig. 1A. Fig. 2, A and B show the mouse gastric mucosal cells reacted with mAbs 18A10 and 10A6, respectively. The signals for IP₃R-1 and -3 were observed throughout the parietal cells except in the nucleus. The reticular distribution pattern suggests that both the IP₃R-1 and IP₃R-3 are present at the membranes of intracellular compartments (probably the endoplasmic reticulum). The immunoreactivity of mAb KM1083 for the IP₃R-2 was not observed in gastric mucosal cells of rat stomach (data not shown).

IP₃R-2 Localized in Goblet Cells of Small Intestine

We observed the immunoreactivity of mAb KM1083 in villus and crypt goblet cells of rat duodenum, jejunum, and ileum. Fig. 3 shows the immunohistochemistry of mAb KM1083 in epithelium of rat duodenum. The mucus of these cells was counterstained with alcian blue (pH 2.5). The immunosignals were distributed throughout the cytoplasm of the cells (Fig. 3A). A control section treated with the same antibody adsorbed with the peptide antigen gave no immunostaining (Fig. 3B). It was therefore concluded that IP₃R-2 resides predominantly in the goblet cells of small intestine. Goblet cells in colon were not immunostained for the IP₃R-2, although their mucus was also reacted with alcian blue (pH 2.5) reagent (Fig. 3C).

Fig. 3 Immunocytochemical localization of IP₃R-2 in mucosal villi of rat duodenum (A and B) and mucosa of rat colon (C). Tissue sections (10 μ m thick) were incubated with mAb KM1083 (A and C) or mAb KM1083 pre-incubated with the immunogen peptide (B). The sections were counterstained with alcian blue (pH 2.5). Scale marker, 25 μ m

Western Blotting Analysis

This was performed to verify the immunolocalizations of the IP₃R types described above. It is not

Fig. 4 Western blotting analysis of three types of IP₃R in rat gastrointestinal. Fifty μ g of membrane proteins of various gastrointestinal tissues were reacted with mAbs KM1112 (A), KM1083 (B) and KM1082 (C), respectively. Lanes 1 to 10 were; esophagus, cardia of stomach, body of stomach, pylorus of stomach, duodenum, jejunum, ileum, caecum, colon and rectum. Sequentially, right lanes of 1 to 10 represent analysis by the mAbs pre-incubated with the immunogen peptides. Arrows in (A), (B) and (C) indicate the bands of the IP₃R types. The lower molecular weight bands seem to be degraded proteins of IP₃Rs.

possible to quantitate the expression levels of the IP₃Rs in the epithelial cells of gastrointestinal, because each of the protein samples contains submucosa and muscularis of gastrointestinal tract; these muscle cells also exhibit characteristic expression of the three IP₃R types. However, immunoreactive bands of approximately 250 kDa were clearly detected in stomach for IP₃R-1 and IP₃R-3 (Fig. 4, A and C, lanes 2–4) and in small intestine for IP₃R-2 (Fig. 4B, lanes 5–7). All the bands disappeared in the presence of the immuno-

gen peptides. The lower molecular weight protein bands were assumed to be degraded IP₃Rs; their intensity was increased when the samples were incubated for 1 h at 4°C before homogenization (data not shown).

DISCUSSION

In the present study, cellular localizations of the three types of IP₃R (IP₃R-1, IP₃R-2 and IP₃R-3) in rat gastrointestinal epithelial tissues were deter-

mined by immunohistochemistry. We used type-specific monoclonal antibodies KM1112, KM1083, and KM1082, which recognize IP₃R-1, IP₃R-2 and IP₃R-3, respectively.

Prominent immunoreactivities for IP₃R-1 and IP₃R-3 were found in gastric parietal cells (Fig. 1). Mouse gastric parietal cells were also immunostained by three rat monoclonal antibodies against the mouse IP₃R-1:mAb 4C11, mAb 10A6, and mAb 18A10. In a previous *in situ* hybridization experiment, we detected the transcripts of IP₃R-3 in the mouse gastric glands (6). These findings strongly support the conclusion that both IP₃R-1 and IP₃R-3 are present in the gastric parietal cells. Recently, Monkawa *et al.* have demonstrated that distinct types of IP₃R subunits assemble to form heterotetramers in Chinese hamster ovary cells (CHO-K1 cells) and rat liver (16). Thus, the colocalization of IP₃R-1 and IP₃R-3 suggests the possibility that the two types of IP₃Rs form a heterotetrameric structure in parietal cells.

The gastric parietal cells reside mainly in the upper half of the gastric gland and produce hydrochloric acid. The secretion of HCl by parietal cells can be activated by different mechanisms. One mode of activation is through cholinergic nerve endings and the transmitter acetylcholine (ACh). Histamine and a polypeptide gastrin, both secreted in the gastric mucosa, also act to stimulate the production of hydrochloric acid (9). Histamine activates adenylate cyclase, resulting in the accumulation of cAMP responsible for gastric acid secretion. Gastrin and ACh enhance inositol phospholipid turnover followed by an increase in the intracellular concentration of Ca²⁺ ([Ca²⁺]_i) (19), probably due to the Ca²⁺ mobilization through the IP₃Rs. Gastrin and ACh are known to potentiate histamine-mediated gastric acid secretion, and the increase in [Ca²⁺]_i might augment adenylate cyclase activity or directly stimulate the secretory machinery. The IP₃R-1 in parietal cells could be phosphorylated by cAMP-dependent protein kinase (PKA), and we recently demonstrated that the phosphorylation enhances the Ca²⁺ channel activity of the IP₃R-1 (18). IP₃R-3 also possesses a few putative phosphorylation sites for PKA (7). The gastric parietal cell would provide a good cell system to investigate the interrelationship (cross talk) between the two second messenger (cAMP and IP₃) systems.

We immunolocalized the IP₃R-2 in the goblet cells of small intestinal villi and cryptae (Fig. 3). Counterstaining with alcian blue (pH 2.5) revealed

that the IP₃R-2 is present in all the goblet cells of small intestine. By contrast, no significant immunoreactivity was observed in the goblet cells of colon. The difference in the immunoreactivity for the IP₃R-2 between small and large intestine might reflect the difference in cell functions. The goblet cells secrete acid glycoprotein which protect and lubricate the lining of the intestine. Inositol phospholipid turnover and subsequent IP₃R-induced Ca²⁺ release might be involved in the intestinal secretory events.

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Note added in proof: The gastric parietal cells of type 1 IP₃R-deficient mice showed no immunoreactivity of the mAbs against mouse type-1 IP₃R. The gastric functions of the deficient mice, including acid secretion are now under investigation.

REFERENCES

- BERRIDGE M. J. (1993) Inositol trisphosphate and calcium signaling. *Nature* **361**, 315–325
- BLONDEL O., TAKEDA J., HEILWIG J., SEINO S. and BELL G. I. (1993) Sequence and functional characterization of a third inositol trisphosphate receptor subtype, IP₃R-3, expressed in pancreatic islets, kidney, gastrointestinal tract, and other tissues. *J. Biol. Chem.* **268**, 11356–11363
- BOZOU J. C., ROCHET N., MAGNALDO I., VINCENT J. P. and KITABGI P. (1989) Neurotension stimulates inositol trisphosphate-mediated calcium mobilization but not protein kinase C activation in HT29 cells, involvement of a G-protein. *Biochem. J.* **264**, 871–878
- BUSH K. T., STUART R. O., LI S. H., MOURA L. A., SHARP A. H., ROSS C. A. and NIGAM S. K. (1994) Epithelial inositol 1,4,5-trisphosphate receptors, multiplicity of localization, solubility, and isoforms. *J. Biol. Chem.* **269**, 23694–23699
- DICKINSON K. E., FRIZZELL R. A. and SEKAR M. C. (1992) Activation of T84 cell chloride channels by carbachol involves a phosphoinositide-coupled muscarinic M₃ receptor. *Eur. J. Pharmacol.* **225**, 291–298
- FUJINO I., YAMADA N., MIYAWAKI A., HASEGAWA M., FURUICHI T. and MIKOSHIBA K. (1995) Differential expression of type 2 and type 3 inositol 1,4,5-trisphosphate receptor mRNAs in various mouse tissues: *in situ* hybridization study. *Cell Tissue Res.* **280**, 201–210
- FURUICHI T., KOHDA K., MIYAWAKI A. and MIKOSHIBA K. (1994) Intracellular channels. *Curr. Opin. Neurobiol.* **4**,

- 294–303
8. FURUICHI T., YOSHIKAWA S., MIYAWAKI A., WADA K., MAEDA N. and MIKOSHIBA K. (1989) Primary structure and functional expression of the inositol 1,4,5-trisphosphate-binding protein P₄₀₀. *Nature* **342**, 32–38
 9. HIRSCHOWITZ B. I., KEELING D., LEWIN M., OKABE S., PARSONS M., SEWING K., WALLMARK B. and SACHS G. (1995) Pharmacological aspects of acid secretion. *Dig. Dis. Sci.* **40**, 3S–23S
 10. LIEBERHERR M., GROSSE B., DUCHAMBON P. and DRUEKE T. (1989) A functional cell surface type receptor is required for the early action of 1,25-dihydroxyvitamin D₃ on the phosphoinositide metabolism in rat enterocytes. *J. Biol. Chem.* **264**, 20403–20406
 11. MAEDA N., NIINOBE M., INOUE Y. and MIKOSHIBA K. (1989) Developmental expression and intracellular localization of P₄₀₀ protein characteristic of Purkinje cells in the mouse cerebellum. *Dev. Biol.* **133**, 67–76
 12. MAEDA N., NIINOBE M., NAKAHIRA K. and MIKOSHIBA K. (1988) Purification and characterization of P₄₀₀ protein, a glycoprotein characteristic of Purkinje cell from mouse cerebellum. *J. Neurochem.* **51**, 1724–1730
 13. MARANTO A. R. (1994) Primary structure, ligand binding, and localization of the human type 3 inositol 1,4,5-trisphosphate receptor expressed in intestinal epithelium. *J. Biol. Chem.* **269**, 1222–1230
 14. MIGNERY G. A., NEWTON C. L., ARCHER B. T. and SÜDHOF T. C. (1990) Structure and expression of the rat inositol 1,4,5-trisphosphate receptor. *J. Biol. Chem.* **265**, 12679–12685
 15. MIGNERY G. A., SÜDHOF T. C., TAKEI K. and DE CAMILLI P. (1989) Putative receptor for inositol 1,4,5-trisphosphate similar for ryanodine receptor. *Nature* **342**, 192–195
 16. MONKAWA T., MIYAWAKI A., SUGIYAMA T., YONESHIMA H., YAMAMOTO-HINO M., FURUICHI T., SARUTA T., HASEGAWA M. and MIKOSHIBA K. (1995) Heterotetrameric complex formation of inositol 1,4,5-trisphosphate receptor. *J. Biol. Chem.* (in press)
 17. MORRIS A. P. and FRIZZELL R. A. (1993) Ca²⁺-dependent Cl[−] channels in undifferentiated human colonic cells (HT-29), I. single-channel properties. *Amer. J. Physiol.* **264**, C968–C976
 18. NAKADE S., RHEE S. K., HAMANAKA H. and MIKOSHIBA K. (1994) Cyclic AMP-dependent phosphorylation of an immunoaffinity-purified homotetrameric inositol 1,4,5-trisphosphate receptor (type 1) increases Ca²⁺ flux in reconstituted lipid vesicles. *J. Biol. Chem.* **269**, 6735–6742
 19. ROCHE S., BALI J. P., GALLEYRAND J. C. and MAGOUS R. (1991) Characterization of a gastrin-type receptor on rabbit gastric parietal cells using L365,260 and L364,718. *Amer. J. Physiol.* **260**, G182–G188
 20. ROSS C. A., DANOFF S. K., SCHELL M. J. and SNYDER S. H. (1992) Three additional inositol 1,4,5-trisphosphate receptors: molecular cloning and differential localization in brain and peripheral tissues. *Proc. Natl. Acad. Sci. USA* **89**, 4265–4269
 21. SÜDHOF T. C., NEWON C. L., ARCHER B. T., USHKARYOV Y. A. and MIGNERY G. A. (1991) Structure of a novel InsP₃R receptor. *EMBO J.* **10**, 3199–3206
 22. SUGIYAMA T., FURUYA A., MONKAWA T., YAMAMOTO-HINO M., SATOH S., OHMORI K., MIYAWAKI A., HANAI N., MIKOSHIBA K. and HASEGAWA M. (1994) Monoclonal antibodies distinctively recognizing the subtypes of inositol 1,4,5-trisphosphate receptor: application to the studies on inflammatory cells. *FEBS Lett.* **354**, 149–154
 23. YAMADA N., MAKINO Y., CLARK R. A., PEARSON D. W., MATTEI M. G., GUÉNET J. L., OHAMA E., FUJINO I., MIYAWAKI A., FURUICHI T. and MIKOSHIBA K. (1994) Human inositol 1,4,5-trisphosphate type 1 receptor, InsP₃R1: structure, function, regulation of expression and chromosomal localization. *Biochem. J.* **302**, 781–790
 24. YAMAMOTO-HINO M., SUGIYAMA T., HIKICHI K., SEKINE S., SAKURADA K., MIYAWAKI A., FURUICHI T., HASEGAWA M. and MIKOSHIBA K. (1994) Cloning and characterization of human inositol 1,4,5-trisphosphate receptor type 2 and type 3. *Receptors and Channels* **2**, 9–22