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Region-specific distribution of the $P2Y_4$ receptor in enteric glial cells and interstitial cells of Cajal within the guinea-pig gastrointestinal tract

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Abstract

Although there is pharmacological evidence to assume that the $P2Y_4$ receptor is a regulator of epithelial ion transport, no detailed data about its distribution within the gut are available. Therefore, this study, using whole mounts and cryosections, aimed to reveal the expression pattern of $P2Y_4$ along the entire guinea-pig gastrointestinal tract. $P2Y_4$ immunoreactivity was absent from enteric neurons but present in enteric glial cells of the stomach, small and large intestine. In the esophagus, $P2Y_4$ appeared to be exclusively located within striated muscle cells. $P2Y_4$ showed also a region dependency regarding its presence in different subpopulations of interstitial cells of Cajal: in myenteric interstitial cells of Cajal in the stomach and ileum; in some intramuscular interstitial cells in the stomach and cecum; in some deep muscular plexus interstitial cells in the ileum; and in some submucosal surface interstitial cells in the colon. These results and the knowledge that $P2Y_4$ activation causes intracellular Ca^{2+} recruitment led us to suggest that $P2Y_4$ in enteric glia plays a modulatory role in intercellular Ca^{2+} waves, while $P2Y_4$ in interstitial cells of Cajal modulates intracellular Ca^{2+} oscillations.

Keywords: $P2Y_4$ receptor; Gastrointestinal tract; Interstitial cell of Cajal; Enteric glia

1. Introduction

Adenine and uridine nucleotides are present in all types of cells and are released in response to various stimuli. Once in the extracellular space, they are able to activate membrane proteins, which are categorized as P2 receptors, comprising ionotropic P2X and metabotropic P2Y receptors. P2Y receptors are characterized by seven putative transmembrane domains typical of G protein-coupled receptors. At present, 15 heptahelical proteins have been associated with the P2Y receptor family. However, only eight P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) are accepted as clearly defined, distinct, nucleotide receptors. P2Y receptors can be broadly subdivided into G_q-coupled subtypes ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{11}$) and G_i-coupled subtypes ($P2Y_{12}$, $P2Y_{13}$ and $P2Y_{14}$) (for review, see King and Burnstock, 2002; Burnstock and Knight, 2004).

The $P2Y_4$ receptor was cloned first from human placenta (Communi et al., 1995) and from genomic human DNA (Nguyen et al., 1995), followed by cloning of rat (Bogdanov et al., 1998; Webb et al., 1998) and mouse (Lazarowski et al., 2001; Suarez-Huerta et al., 2001) orthologs. The human $P2Y_4$ receptor is a selective UTP receptor, whereas the rodent ones are equipotently activated by UTP and ATP. $P2Y_4$ mRNA has been detected in the murine stomach and intestine (Suarez-Huerta et al., 2001; Robaye et al., 2003), in murine colonic crypts (Matos et al., 2005), and in rat (Christofi et al., 2004) and guinea-pig (Cooke et al., 2004) colonic submucosa. Pharmacological studies have provided evidence that the $P2Y_4$ receptor is a dominant nucleotide-

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sensitive regulator of salt and fluid transport in the intestine (Cressman et al., 1999; Robaye et al., 2003; Christofi et al., 2004; Ghanem et al., 2005; Matos et al., 2005). Although some morphological studies have been performed to reveal the distribution of the $P2Y_4$ receptor in the gut, the distribution of this receptor in the gastrointestinal (GI) tract remains obscure. In archival, paraffin-embedded human bowel tissue, $P2Y_4$ immunoreactivity (IR) has been localized in enteric neurons and in a subpopulation of epithelial cells (Clunes et al., 2002). $P2Y_4$ immunostaining has also been observed in a few submucosal enteric neurons in the rat (Christofi et al., 2004) and guinea-pig (Cooke et al., 2004) distal colon. A more recent study reported that the $P2Y_4$ receptor was expressed in enteric glial cells (EGCs) and in some interstitial cells of Cajal (ICCs) in the rat distal colon (Van Nassauw et al., 2005). However, an immunocytochemical study attempting to determine the distribution pattern of $P2Y_4$ receptors in the murine GI tract failed to demonstrate IR for this purinoceptor along the mouse gut (Giaroni et al., 2002).

Given the current lack of knowledge about the GI expression of the $P2Y_4$ receptor, the present study was performed to disclose the distribution of the $P2Y_4$ receptor in the GI tract to better understand how this receptor is involved in intrinsic control of GI functions and to provide a more detailed morphological substrate for the pharmacological data.

2. Materials and methods

Tissue was obtained from adult guinea-pigs ($n=5$, weight=ca. 350 g) of both genders (Charles River Laboratories, Brussels, Belgium). All animals had free access to water and complete rodent diet and were kept in a 12-h/12-h light/dark cycle. The animals were sacrificed by cranial concussion. All procedures were approved by the local ethics committee of the University of Antwerp.

Different regions of the guinea-pig GI tract (esophagus, gastric fundus and corpus, ileum, cecum, proximal and distal colon) were removed and rinsed in a Krebs solution (117 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2.2H_2O$, 1.2 mM $MgSO_4{\cdot}7H_2O$, 25 mM $NaHCO_3$, 1.2 mM $NaH_2PO_4{\cdot}2H_2O$ and 10 mM glucose, pH 7.4). At the distal end of each collected tissue part, a 3-mm segment was removed and processed for cryosectioning. These segments were immersed in fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.0) for 3 h at room temperature. Next, they were rinsed in PBS, transferred to PBS containing 20% sucrose and stored at 4 °C overnight. Ultimately, they were embedded in OCT-embedding medium, sectioned at 10 μm and thaw-mounted on gelatine-coated slides.

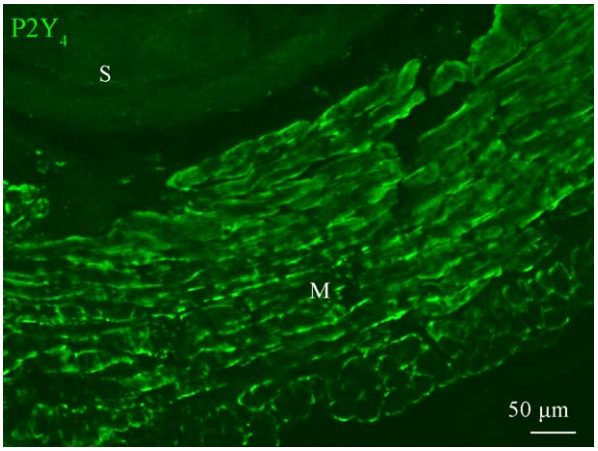

Fig. 1. In the esophagus, $P2Y_4$ IR was only detected in the cell membrane of striated muscle cells. M: outer muscle layer, S: submucosa.

The remaining parts of the collected tissue segments were processed for whole-mount preparation. The gastric segments were opened along both curvatures, and the segments of esophagus, ileum and colon were opened along the mesenteric border. All opened tissue segments were pinned out on a Sylgard-lined Petri dish filled with the above-mentioned fixative for 3 h at room temperature. Subsequently, they were rinsed in 0.01 M phosphate-buffered saline (PBS, pH 7.4) and further processed to optimize the immunocytochemical staining as previously described (Llewellyn-Smith et al., 1985). Following fixation

Table 1
List of antisera used for immunocytochemistry

Antigen	Host	Dilution	Source
Primary antisera			
$P2Y_4$ receptor	Rabbit	1:500	Alomone Labs Ltd., Jerusalem, Israel (APR-006)
c-Kit protein (Kit)	Goat	1:100	Santa Cruz Biotechnology, Santa Cruz, CA (sc-1494)
S100	Rabbit	1:1000	DAKO A/S, Glostrup, Denmark (Z0311)
Protein gene product 9.5 (PGP)	Rabbit	1:4000	Biogenesis, Poole, UK (7863-0504)
Secondary antisera and streptavidin complexes			
Cy3-conjugated goat anti-rabbit IgG		1:4000	Jackson ImmunoResearch Laboratories, West Grove, PA
Biotinylated swine anti-rabbit IgG		1:400	DAKO A/S
Cy3-conjugated donkey anti-goat		1:200	Jackson ImmunoResearch Laboratories
FITC-conjugated streptavidin		1:1000	Jackson ImmunoResearch Laboratories
ExtrAvidin-horseradish peroxidase (HRP)		1:1000	Sigma
Biotin-conjugated tyramide signal amplification kit			PerkinElmer Life Sciences, Boston, MA

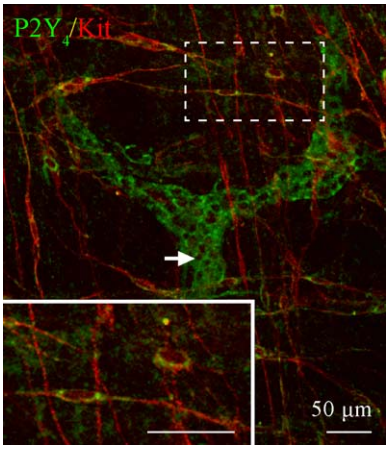

Fig. 2. Double immunolabelling with antibodies directed against $P2Y_4$ and Kit. In the gastric corpus, most myenteric Kit-immunostained ICCs expressed $P2Y_4$ IR. Based on double immunostaining with antibodies directed against $P2Y_4$ and S100, it was concluded that Kit-negative cells expressing $P2Y_4$ IR are EGCs. Arrow: $P2Y_4$-expressing EGCs in a myenteric ganglion. Inset: a higher magnification (bar = 50 µm) of the white dotted box, showing myenteric Kit-immunopositive ICCs expressing $P2Y_4$ IR.

and clearing, the tissues were dissected into two layers: the outer musculature with adhering serosa and the submucosa/mucosa. The circular muscle layer of the intestinal and gastric outer musculature, and the inner part of the esophageal striated muscle layer were partially removed. The mucosa in the intestinal whole mounts containing the submucosal layer was scraped off using a blunt scalpel. The gastric and esophageal submucosal whole mounts were not analyzed in this study. The whole mounts were stored in PBS containing 0.1% sodium azide.

All immunocytochemical incubations were carried out at room temperature as previously described (Van Nassauw et al., 2002). Unless indicated otherwise, washes with PBS were performed between each incubation step. The antisera and streptavidin complexes used in this study as well as their respective working dilutions are listed in Table 1.

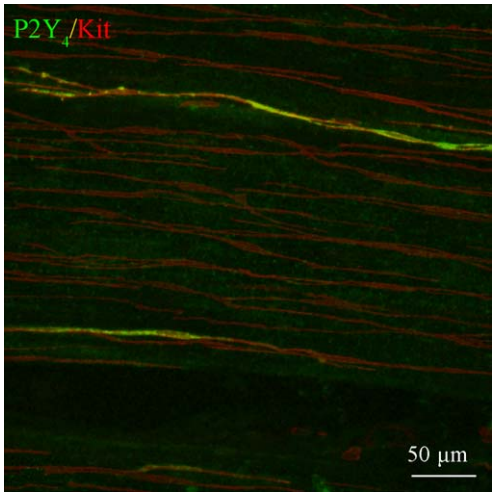

Fig. 3. Double immunolabelling with antibodies directed against $P2Y_4$ and Kit. Some intramuscular ICCs in the gastric corpus expressed $P2Y_4$ IR.

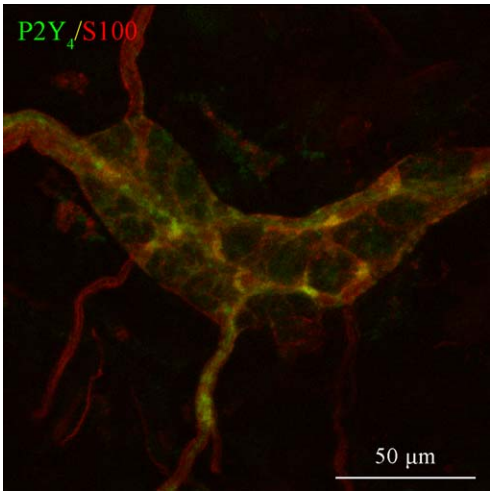

Fig. 4. Double immunolabelling with antibodies directed against $P2Y_4$ and S100. In the submucosal plexus of the ileum, $P2Y_4$ IR was detected in EGCs.

In single- and double-labelling experiments, the $P2Y_4$ receptor was always visualized using the biotin-conjugated tyramide signal amplification (TSA) technique, which was modified for whole mounts (Van Nassauw et al., 2002). Briefly, to minimize endogenous peroxidase activity, the cryosections and whole mounts were treated with 3% hydrogen peroxide in methanol. Next, they were immersed in 0.1 M PBS (pH 7.4) containing 0.05% thimerosal (PBS*), 10% normal horse serum (NHS) and 1% Triton X-100, before incubation for 18 h (cryosections) or 48 h (whole mounts) with a polyclonal antibody directed against the $P2Y_4$ receptor, diluted in PBS* containing 10% NHS and 0.1% Triton X-100. This anti-$P2Y_4$ antibody was directed against a synthetic peptide corresponding to residues 337–350 of the C-terminal part of the rat $P2Y_4$ receptor. The cryosections and whole mounts were consecutively incubated with biotinylated swine anti-rabbit IgG diluted in

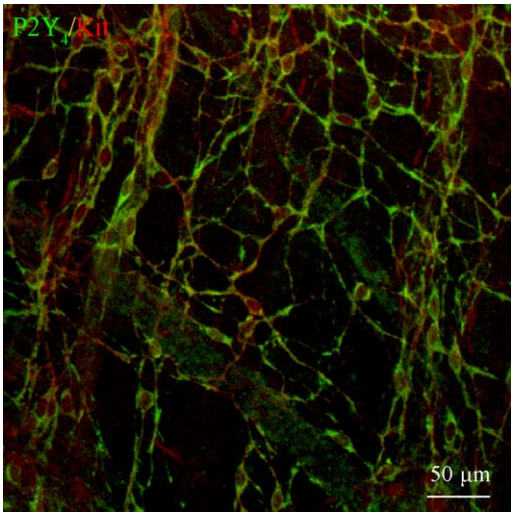

Fig. 5. Double immunolabelling with antibodies directed against $P2Y_4$ and Kit. $P2Y_4$ immunostaining was detected in most myenteric ICCs.

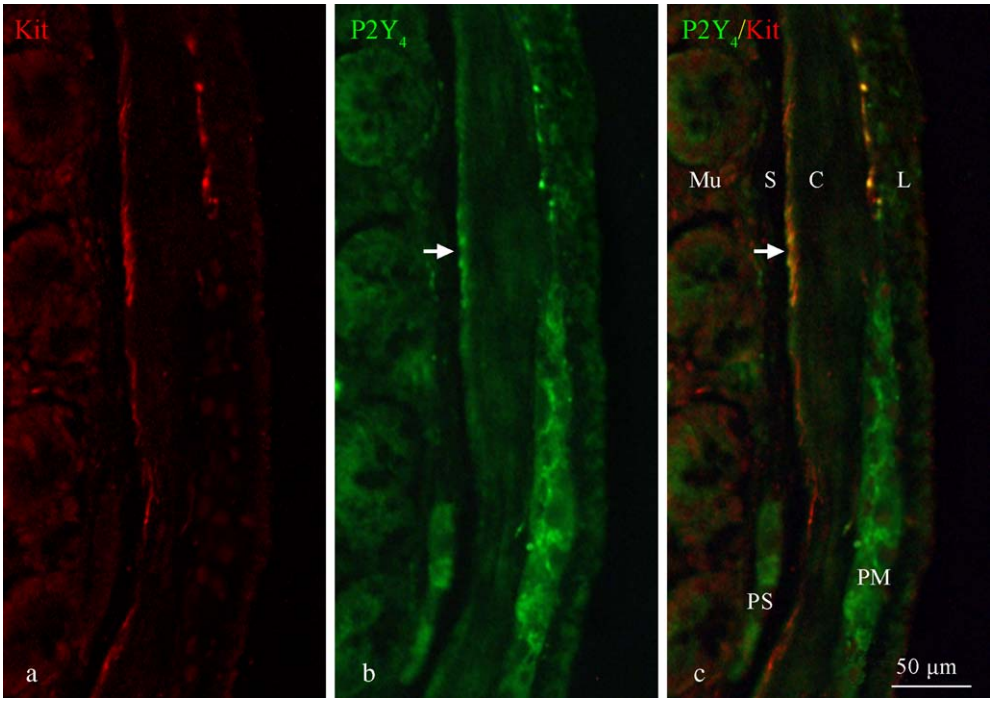

Fig. 6. Double immunolabelling with antibodies directed against Kit (a) and $P2Y_4$ (b). $P2Y_4$ IR was not only found in EGCs and in myenteric ICCs, but also in some deep muscular plexus ICCs (arrow). (c) Merged image of (a) and (b). C: circular muscle layer, L: longitudinal muscle layer, S: submucosa, Mu: mucosa, PS: submucosal plexus, PM: myenteric plexus.

PBS* containing 1% NHS and with ExtrAvidin-horseradish peroxidase diluted in PBS*. Between subsequent steps, the whole mounts and cryosections were washed in PBS containing 0.05% Tween 20. Whole mounts were then incubated for 20 min in biotin-conjugated tyramide diluted in an amplification solution. Visualization was performed using fluorophore-conjugated streptavidin diluted in PBS*. After washing, the cryosections and whole mounts were mounted in Citifluor.

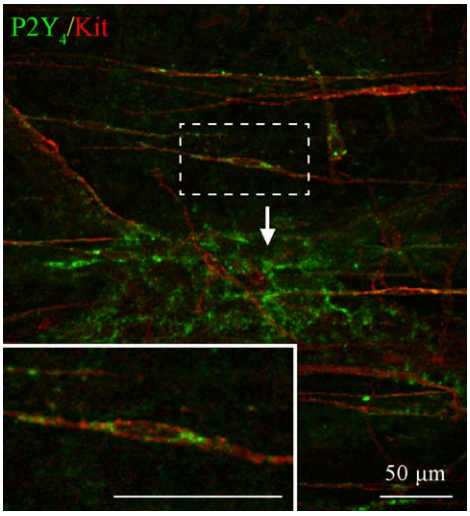

Fig. 7. Double immunolabelling with antibodies directed against $P2Y_4$ and Kit. In the cecum, $P2Y_4$ was expressed in many Kit-immunoreactive intramuscular ICCs. Based on double immunostaining with antibodies directed against $P2Y_4$ and S100, it was concluded that Kit-negative cells expressing $P2Y_4$ IR are EGCs. Arrow: $P2Y_4$-expressing EGCs in a myenteric ganglion. Inset: a higher magnification (bar=50 µm) of the white dotted box, showing a Kit-immunopositive intramuscular ICC expressing $P2Y_4$ IR.

Double-labelling experiments, using primary antisera raised in different species, were conducted with the indirect immunofluorescence method. Using two primary antisera raised in the same species, a sequential immunostaining procedure was performed according to the method of Shindler and Roth (1996). In the first step, the TSA technique was used to detect the $P2Y_4$ receptor, while in the second step a conventional immunofluorescence method was applied. For the second step, we used antibodies (Table 1) directed against protein gene product 9.5 (PGP), which labels most enteric neurons (Phillips et al., 2004), against S100, a marker of EGCs (Jessen and Mirsky, 1985), and against c-kit protein (Kit), a marker of ICCs (Burns et al., 1997). Negative controls in which one of the primary antibodies was omitted and interference control stainings, as described by Shindler and Roth (1996), were performed. The specificity of the anti-$P2Y_4$ antibody was tested by preabsorption of the antibody (1 µg antibody/100 µl PBS) with the homologous peptide antigen (2 µg antigen/100 µl PBS) (Alomone Labs Ltd.). The whole-mount preparations and cryosections were evaluated with fluorescence and confocal microscopy.

3. Results

In the guinea-pig GI tract, $P2Y_4$ IR was observed in each GI segment under study. Control immunostainings, in which

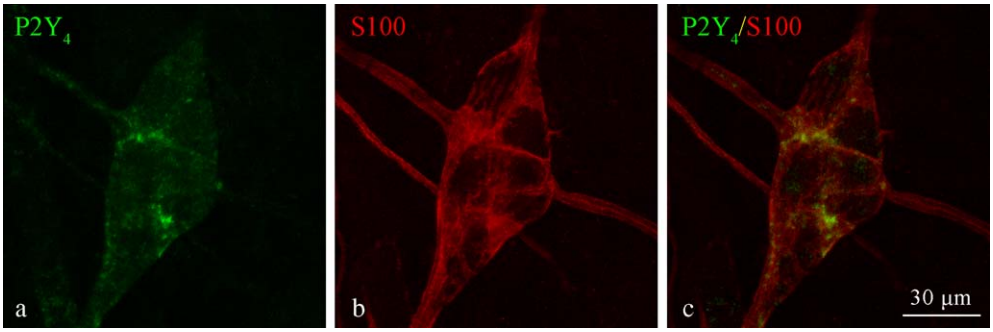

Fig. 8. Double immunolabelling with antibodies directed against $P2Y_4$ (a) and S100 (b) in the submucosal plexus of the proximal colon showing the expression of $P2Y_4$ in EGCs. (c) Merged image of (a) and (b).

the primary antibody was omitted or preabsorbed, did not yield immunolabelling. Interference control stainings showed no linking of secondary antibodies with primary antibodies used in previous steps. In the esophagus, immunostaining for the $P2Y_4$ receptor was only observed in the cell membrane of striated muscle cells (Fig. 1). No other cell types expressed $P2Y_4$ IR. In general, both EGCs and ICCs, but not enteric neurons, expressed $P2Y_4$ IR within the gastric, small intestinal and cecal regions, while immunostaining in the colon was predominantly restricted to EGCs.

In both the gastric fundus and corpus, immunostaining was observed in EGCs and in almost all myenteric ICCs (Fig. 2). Some intramuscular ICCs also appeared to bear the $P2Y_4$ receptor (Fig. 3). In the submucosal layer of the ileum, $P2Y_4$ IR was only detected in EGCs located in the submucosal ganglionic nerve network (Figs. 4 and 6), whereas at the level of the outer muscle layer, the $P2Y_4$ receptor was expressed in virtually all myenteric ICCs (Figs. 5 and 6), in some ICCs of the deep muscular plexus and in all EGCs (Fig. 6). In the wall of the cecum, which lacks myenteric ICCs (Burns et al., 1997), EGCs and most intramuscular ICCs were immunolabelled (Fig. 7). In the submucosa of the proximal and distal colon, enteric glia (Fig. 8) and some submucosal surface ICCs (Fig. 9) bore the $P2Y_4$ receptor. Furthermore, in the outer muscle layer, $P2Y_4$ IR was only found in EGCs (Fig. 9). In the distal colon, a few $P2Y_4$-expressing myenteric ICCs were detected. No specific immunostaining was observed in the mucosa throughout the GI tract.

4. Discussion

Previous studies in mouse and rat indicate that ATP and UTP modulate electrolyte transport in the small and large intestine, and that these responses are mediated by P2Y receptors, including the $P2Y_4$ receptor (Cressman et al., 1999; Robaye et al., 2003; Christofi et al., 2004; Ghanem et al., 2005; Matos et al., 2005). Although $P2Y_4$ expression has been demonstrated in the GI tract (mouse: Suarez-Huerta et al., 2001; Robaye et al., 2003; Matos et al., 2005; rat: Christofi et al., 2004; guinea-pig: Cooke et al., 2004), the data on the distribution of this receptor in the gut are incomplete and contradictory. Therefore, we aimed to elucidate the expression of the $P2Y_4$ receptor in the GI tract. The guinea-pig was selected for this study because the neurochemistry of the enteric nervous system in this mammal has been studied quite elaborately in most detail and because a previous study has reported on the expression of the $P2Y_4$ receptor in guinea-pig enteric neurons (Cooke et al., 2004). The present results are the first data establishing $P2Y_4$ expression in specific non-neuronal cell types throughout the guinea-pig GI tract. Except for the esophagus, $P2Y_4$ IR was found in EGCs. The myenteric ICCs in

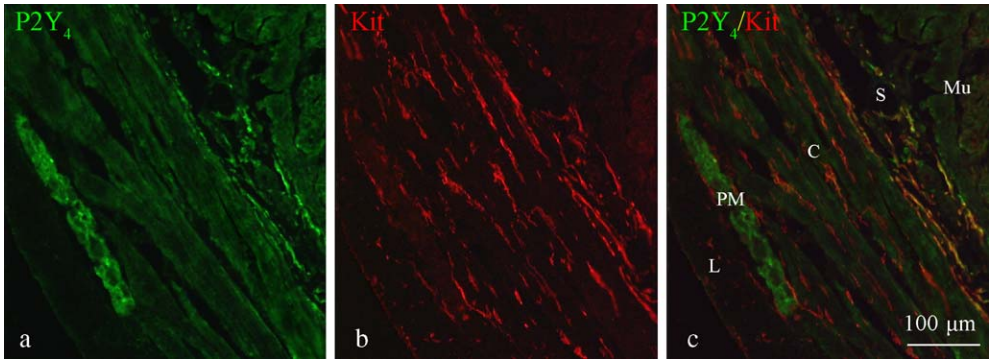

Fig. 9. Double immunolabelling with antibodies directed against $P2Y_4$ (a) and Kit (b) in the guinea-pig proximal colon. $P2Y_4$ IR was detected in EGCs and in some submucosal surface ICCs. $P2Y_4$ was not found in intramuscular and myenteric ICCs. (c) Merged image of (a) and (b). C: circular muscle layer, L: longitudinal muscle layer, S: submucosa, Mu: mucosa, PM: myenteric plexus.

the small intestine and the stomach, some intramuscular ICCs in the stomach and cecum, some ICCs of the deep muscular plexus of the small intestine and some submucosal surface ICCs in the colon, also appeared to express the $P2Y_4$ receptor.

Enteric glia represent the most abundant cells in the enteric nervous system (ENS). The traditional assumption that EGCs serve only as supportive or nutritive elements for enteric neurons is currently being challenged. EGCs appear to be actively involved in the regulation of neuronal activity within the ENS. They are important regulators of mucosal permeability and barrier function, and of epithelial cell growth in the GI tract. They actively participate in the course of intestinal inflammation (for review see: Cabarrocas et al., 2003; Rühl et al., 2004; Rühl, 2005). EGCs are capable of transmitting increases in intracellular Ca^{2+} to surrounding EGCs. These so-called intercellular Ca^{2+} waves involve a sequence of intracellular and extracellular steps in which phospholipase C (PLC), inositol triphosphate (IP_3) and ATP play essential roles. It has been suggested that EGCs bear P2Y receptors activating PLC and promoting IP_3 signalling (Kimball and Mulholland, 1996; Zhang et al., 2003). Activation of the $P2Y_4$ receptor results in responses signalling through the PLC/IP_3/Ca^{2+} pathway (King and Burnstock, 2002; Burnstock and Knight, 2004). Similar to the rat distal colon (Van Nassauw et al, 2005), we could demonstrate the presence of the $P2Y_4$ receptor in EGCs within the guinea-pig stomach and intestines. Other studies revealed that the $P2Y_4$ receptor is also expressed in other glial cell types–viz. in astrocytes (Lenz et al., 2000; Suadicani et al., 2004), satellite glial cells (Weick et al., 2003) and Müller glial cells (Fries et al., 2005)–and in microglia, which are small glial cells related to phagocytic cells (Bianco et al., 2005). Other P2 receptors have also been demonstrated in EGCs: the $P2Y_6$ receptor in the rat distal colon (Van Nassauw et al., 2005) and $P2X_7$ subunits in the rat GI tract (Vanderwinden et al., 2003). The present data point to an involvement of $P2Y_4$ in intercellular Ca^{2+} waves in the enteric glial network, as previously reported in astrocytes (Suadicani et al., 2004).

ICCs constitute a number of subpopulations with common morphological features but different functions, including generation of gut pacemaker activity, transmission of nerve signals to smooth muscle and sensing of mechanical activity (for review, see Huizinga et al., 2004; Ward et al., 2004). It has been shown that myenteric ICCs of the guinea-pig ileum express the $P2X_2$ and $P2X_5$ receptors and that $P2X_5$ receptors have been demonstrated on myenteric ICCs of the murine ileum (Burnstock and Lavin, 2002). $P2X_2$ subunits were also found on ICC-like cells in the vas deferens of rodents (Burton et al., 2000). In the rat distal colon, $P2Y_4$ IR has been detected in some submucosal surface ICCs and in a few myenteric ICCs (Van Nassauw et al., 2005). At present, we demonstrate in the guinea-pig GI tract that the expression of the $P2Y_4$ receptor in the different ICC types is region-specific. Most myenteric ICCs in the stomach and the small intestine, some intramuscular ICCs in the stomach and some ICCs of the deep muscular plexus in the small intestine were immunostained. In the cecum, most intramuscular ICCs expressed $P2Y_4$ IR. Similar to observations made in the rat distal colon (Van Nassauw et al., 2005), $P2Y_4$ IR was found in some submucosal surface ICCs in the guinea-pig colon. A recent pharmacological study has shown that the intracellular Ca^{2+} oscillations during pacemaker activity of ICCs in the murine ileum are modulated by multiple P2 receptors (Furuzono et al., 2005). Our present morphological data are in line with the recently proposed hypothesis that $P2Y_4$ expression in ICCs might be associated with intracellular Ca^{2+} recruitment, because $P2Y_4$ couples via the G_q protein/PLC pathway to cause IP_3 production and intracellular Ca^{2+} mobilization (King and Burnstock, 2002; Burnstock and Knight, 2004).

P2Y receptors are regulators of ion channels, adapting the responsiveness of cells to changes in environmental conditions. These interactions between P2Y receptors and ion channels have been mainly studied in isolated neurons (for review see: Lechner and Boehm, 2004). Given this known modulation of ion channels by P2Y receptors, $P2Y_4$ affecting the excitability state of $P2Y_4$-expressing EGCs and ICCs may indirectly modulate the activity of enteric neurons, including neuromodulatory regulation of epithelial electrolyte transport.

Although in mouse and rat, there is pharmacological evidence to assume expression of the $P2Y_4$ receptor as a specific regulator of epithelial ion transport in GI epithelial cells (Cressman et al., 1999; Robaye et al., 2003; Christofi et al., 2004; Ghanem et al., 2005; Matos et al., 2005), we detected no immunostaining in epithelial cells. This discrepancy may be due to a very low expression of the $P2Y_4$ receptor or to species differences. However, a growing body of evidence indicates that EGCs play a role in GI epithelial cell function. EGC bodies and processes extend to the plexus mucosus and come into close contact with the epithelial cell layer (for review, see Rühl, 2005). So, it is conceivable that $P2Y_4$-expressing EGCs are involved in the modulation of ion transport in GI epithelial cells.

In summary, the present study reveals that the $P2Y_4$ receptor is expressed in specific non-neuronal cell types throughout the guinea-pig GI tract. Whereas EGCs express $P2Y_4$ IR throughout the GI stomach and intestines, the presence of $P2Y_4$ in the different ICC types is region-specific. Expression of $P2Y_4$ in EGCs points to a participation in the modulation of intercellular Ca^{2+} waves in the enteric glial network, while the presence of $P2Y_4$ in ICCs suggests a putative role of this receptor in the regulation of intracellular Ca^{2+} oscillations.

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