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ORIGINAL PAPER

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Distribution of $P2Y_2$ receptors in the guinea pig enteric nervous system and its coexistence with $P2X_2$ and $P2X_3$ receptors, neuropeptide Y, nitric oxide synthase and calretinin

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Abstract The distribution of $P2Y_2$ receptor-immunoreactive (ir) neurons and fibers and coexistence of $P2Y_2$ with $P2X_2$ and $P2X_3$ receptors, neuropeptide Y (NPY), calretinin (CR), calbindin (CB) and nitric oxide synthase (NOS) was investigated with immunostaining methods. The results showed that $P2Y_2$-ir neurons and fibers were distributed widely in myenteric and submucous plexuses of the guinea pig stomach corpus, jejunum, ileum and colon. The typical morphology of $P2Y_2$-ir neurons was a long process with strong positive staining on the same side of the cell body. The $P2Y_2$-ir neurons could be Dogiel type 1. About 40–60% $P2X_3$-ir neurons were immunoreactive for $P2Y_2$ in the myenteric plexus and all the $P2X_3$-ir neurons expressed the $P2Y_2$ receptor in the submucosal plexus; almost all the NPY-ir neurons and the majority of CR-ir neurons were also immunoreactive for $P2Y_2$, especially in the myenteric plexus of the small intestine; no $P2Y_2$-ir neurons were immunoreactive for $P2X_2$ receptors, CB and NOS. It is shown for the first time that S type/Dogiel type 1 neurons with fast P2X and slow P2Y receptor-mediated depolarizations could be those neurons expressing both $P2Y_2$-ir and $P2X_3$-ir and that they are widely distributed in myenteric and submucosal plexuses of guinea pig gut.

Keywords $P2Y_2$ receptor · Enteric nervous system · Guinea pig

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Introduction

The P2-receptor family includes P2X receptors equivalent to intrinsic calcium-permeable cation channels and metabotropic P2Y receptors that are G-protein-coupled receptors (Ralevic and Burnstock 1998; Nicholas 2001). Currently, there are seven cloned P2X ($P2X_1$–$P2X_7$) receptor subunits (Khakh et al. 2001) and at least eight P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$–$P2Y_{14}$) (Burnstock 2004). ATP and UTP may be involved in the complex regulation in the enteric nervous system (ENS) via activation of P2 receptors at pre-synaptic or postsynaptic or postjunctional sites in the gut (Burnstock 2001). In the ENS, ATP activates P2Y and P2X receptors to cause slow and fast membrane depolarizations, respectively, in S/type 1 neurons of the submucosal plexus of the guinea pig ileum (Barajas-Lopez et al. 1994; LePard and Galligan 1999; Galligan et al. 2000; Ren et al. 2003; Hu et al. 2003; Monro et al. 2004). Fast and slow calcium transients underlie the fast and slow membrane depolarizations in these neurons (Barajas-Lopez et al. 2000). These data suggest that P2X and P2Y receptors are co-expressed in these neurons.

Immunocytochemical, in situ hybridization and RT-PCR results showed that $P2X_2$, $P2X_3$ and $P2X_7$ receptors subunits were identified in the whole enteric nervous systems of guinea pig, mouse and rat (Hu et al. 2001; Castelucci et al. 2002, 2003; Poole et al. 2002; Van Nassauw et al. 2002; Xiang and Burnstock 2004a, b). The proteins of $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors in submucosal and myenteric plexuses of rat distal colon and the gene products of $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_{12}$ receptors in the submucous plexus were found (Christofi et al. 2004; Cooke et al. 2004). At present there are no morphological data showing P2X and P2Y receptors coexisting in these neurons and no study shows the systematic distribution of P2Y receptors along the whole length of the intestine. In this study, we used single-labeling and double-labeling immunofluorescence methods to study the distribution of $P2Y_2$ receptors and

its coexistence with $P2X_2$ and $P2X_3$ receptors, NPY, calbindin, calretinin and NOS in the stomach corpus, jejunum, ileum and colon of the guinea pig.

Materials and methods

Animals and tissue preparation

Breeding, maintenance and killing of the animals used in this study followed principles of good laboratory animal care and experimentation in compliance with Home Office (UK) regulations covering Schedule One Procedures in accordance with the Animals (Scientific Procedures) Act, 1986, governing the use of animals. Protocols were approved by the local animal ethics committee. Five guinea pigs were used. Animals were killed by asphyxiation with CO_2 and perfused through the aorta with 0.9% NaCl solution and 4% paraformaldehyde in 0.1 mol/L phosphate buffer, pH 7.4. Stomach, jejunum, ileum, proximal and distal colon were dissected out. Immediately after the segments of the digestive tract were removed, the contents of the lumen were removed with saline. One end of the segment was knotted with a silk thread and fixative was injected into the lumen to fill it and the open end was also knotted with a silk thread. The fixative-filled segment was then immersed in fixative. This was applied to all segments of the intestine examined, including the stomach, from which the contents were removed via the duodenum which was then closed by a knotted silk thread and filled with fixative from the oesophagus. The oesophagus was then closed such that the stomach resembled an irregular balloon. Once fixed, whole-mount preparations could be prepared from the flattened area of the stomach. Whole-mount preparations were prepared of the myenteric plexus of stomach corpus, jejunum, ileum and distal colon and whole-mount preparations of submucous plexus of jejunum, ileum and distal colon were prepared under a dissection microscope.

Immunocytochemistry

The immunocytochemical method was modified from our previous report (Xiang et al. 1998). The preparations were washed 3×5 min in 0.01 mol/L pH 7.2 phosphate-buffered saline (PBS), then incubated in 1.0% H_2O_2 for 30 min to block endogenous peroxidase. Preparations were pre-incubated in 10% normal horse serum (NHS), 0.2% Triton X-100 in PBS for 30 min, followed by incubation with $P2Y_2$ antibodies (Alomone Labs, Jerusalem, Israel), diluted 1:300 in antibody dilution solution (10% NHS, 0.2% Triton X-100 and 0.4% sodium azide in PBS) overnight at 4°C. Subsequently, the preparations were incubated with biotinylated donkey-anti-rabbit IgG (Jackson ImmunoResearch Laboratories, PA, USA) diluted 1:500 in antibody dilution solution for 1 h at 37°C and then with streptavidin-HRP (Sigma Chemical Co., Poole, UK) diluted 1:1000 in PBS for 1 h at 37°C. Finally, a nickel-intensified diaminobenzidine (DAB) reaction was used to visualize immunoreactivity. All incubations and reactions were separated by 3×10 min washes in PBS. The preparations were mounted, dehydrated, cleared and covered.

The development and specificity of the $P2X_2$ and $P2X_3$ polyclonal antibodies (Roche Palo Alto, CA, USA) has been reported previously (Oglebsby et al. 1999). Simultaneous detection of two antigens by immunostaining usually requires primary antibodies from two different species. A novel double-labeling immunostaining method for detection of two independent antigens using two antibodies from the same species of animals has been described (Teramoto et al. 1998). The principle of the method was that the first antigen is detected by the first primary antibody that is diluted so extensively that it cannot be detected with conventional methods; a highly sensitive tyramide signals amplification (TSA) system is used to identify this antibody; the second antigen is stained with the secondary primary antibody and detected by conventional immunostaining. The following protocol was modified from this protocol. Endogenous peroxidase was blocked by 1% H_2O_2 in PBS for 30 min. The sections were pre-incubated in 10% NHS, 0.2% Triton X-100 in PBS for 30 min, followed by incubation with $P2Y_2$, $P2X_2$ and $P2X_3$ receptor antibodies, diluted 1:2,000 in antibody dilution solution (10% NHS, 0.2% Triton X-100 and 0.4% sodium azide in PBS) overnight at 4°C. Subsequently, the sections were incubated with biotinylated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) at a dilution of 1:500 in PBS containing 1% NHS for 1 h. The sections were then incubated in ExtrAvidin peroxidase (Sigma) diluted 1:1,000 in PBS for 30 min at room temperature. The immunoreactivity was visualized by the TSA Fluorescein system (NEL701, NEN, USA). After visualization the sections were incubated with a second different primary antibodies to $P2X_2$, $P2X_3$ and $P2Y_2$ receptors diluted 1:300 in antiserum dilution solution overnight at 4°C. Subsequently, the sections were incubated with Cy3 conjugated donkey-anti-rabbit (Jackson ImmunoResearch Laboratories) diluted 1:300 in antiserum dilution solution for 1 h at room temperature. All the incubations and reactions were separated by 3×10 min washes in PBS.

The following protocol was used for double-immunostaining of $P2Y_2$ receptors with either calbindin, calretinin, NOS, NPY or PGP9.5 (Ultraclone Ltd., Wellow, Isle of Wight, UK; used as a general neuronal maker). The preparations were washed 3×5 min in PBS, then pre-incubated in antibody dilution solution for 30 min, followed by incubation with $P2Y_2$ antibody diluted 1:300 and NOS antibody (sheep-anti-rat) diluted 1:1,000 or calbindin (mouse-anti-rat; SWANT, Bellinzone, Switzerland) diluted 1:5,000 or calretinin (mouse-anti-rat; SWANT) diluted 1:2,000 or PGP9.5 antibody (mouse anti-rat) diluted 1:6,000 in antibody dilution

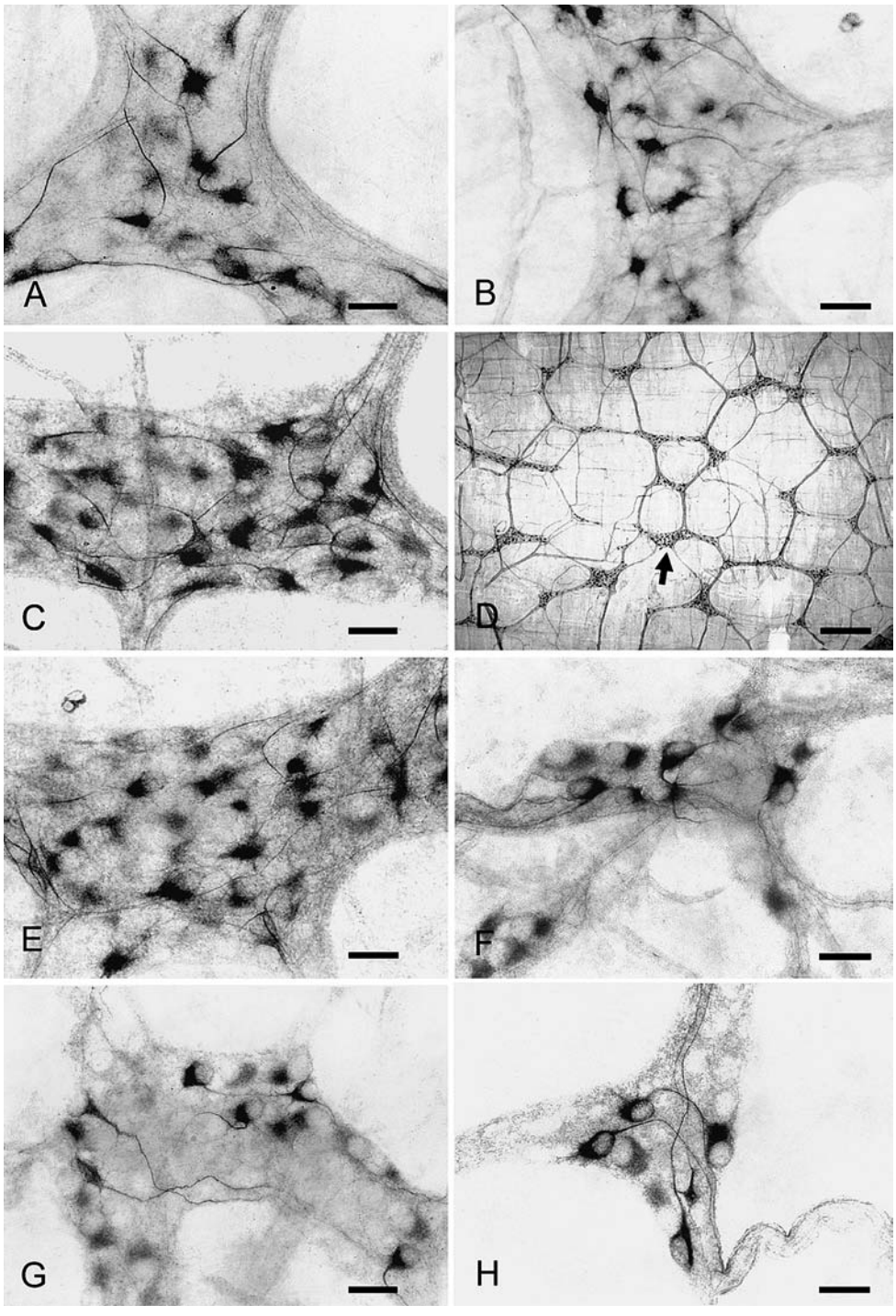

Fig. 1 $P2Y_2$-ir in nerve cell bodies and processes in myenteric and submucosal plexuses of stomach corpus, small intestine and distal colon of adult guinea pig. Most of the positive cells were shown to have a long process with strong positive staining and most of the positive staining was usually seen in the cytoplasm of the side with the positive process, while in the other side the staining was much weaker. **a** $P2Y_2$-ir in nerve cell bodies and processes in myenteric plexus of stomach corpus. **b** $P2Y_2$-ir in nerve cell bodies and processes in myenteric plexus of jejunum. **c** $P2Y_2$-ir in nerve cell bodies and processes in myenteric plexus of ileum. **d** $P2Y_2$-ir in nerve cell bodies and processes in myenteric plexus of distal colon at low magnification. **e** Higher magnification around the area indicated by the arrow. **f** $P2Y_2$-ir in nerve cell bodies and processes in submucosal plexus of jejunum. **g** $P2Y_2$-ir in nerve cell bodies and processes in submucosal plexus of ileum. **h** $P2Y_2$-ir in nerve cell bodies and processes in submucosal plexus of distal colon. *Scale bars* in a and h = 25 μm, in b, c, e, f, g = 50 μm, *scale bar* in d = 250 μm

solution overnight at 4°C. Subsequently, the preparations were incubated with Cy3 conjugated donkey-anti-rabbit IgG (Jackson ImmunoResearch Laboratories) diluted 1:300 diluted for $P2Y_2$ antibodies and FITC conjugated donkey-anti-mouse or sheep IgG (Jackson ImmunoResearch Laboratories) diluted 1:200 in antibody dilution solution for calbindin, calretinin and NOS for 1 h at room temperature. All the incubations and reaction were separated by 3×10 min washes in PBS. The preparations were evaluated with fluorescence microscopy.

Controls

Control experiments were carried out with $P2X_2$, $P2X_3$ and $P2Y_2$ receptor antibodies pre-absorbed with cognate peptide at a concentration of 25 μg/mL.

Photomicroscopy

Images of immunofluorescence labeling were taken with the Leica DC 200 digital camera (Leica, Switzerland)

Table 1 Number of $P2Y_2$ receptor-immunoreactive neurons in the myenteric (*MP*) and submucous plexus (*SMP*) of guinea pig stomach corpus, jejunum, ileum and distal colon, expressed as the mean number of neurons ± SE mean, then expressed as a percentage (the mean number of $P2Y_2$ receptor-immunoreactive neurons was divided by the total number of PGP9.5-positive neurons in the same whole-mount preparations × 100%)

Region	$P2Y_2$-ir	PGP9.5-ir	$P2Y_2$-ir (%)
Stomach corpus MP	181 ± 24	476 ± 42	38 ± 5
Jejunum MP	245 ± 27	454 ± 32	54 ± 7
Jejunum SMP	126 ± 24	297 ± 29	42 ± 6
Ileum MP	286 ± 33	454 ± 51	63 ± 5
Ileum SMP	138 ± 21	300 ± 31	46 ± 9
Distal colon MP	278 ± 29	427 ± 38	65 ± 8
Distal colon SMP	152 ± 19	338 ± 36	45 ± 7

Table 2 Quantitative analysis of double labeling studies between $P2Y_2$ receptors and NPY in the myenteric plexus (MP) and submucous plexus (SMP) of guinea pig stomach corpus, jejunum, ileum and distal colon

Region	$P2Y_2$-ir + NPY-ir +	$P2Y_2$-ir + NPY-ir −	NPY-ir + $P2Y_2$-ir +	NPY-ir + $P2Y_2$-ir −
Stomach corpus MP	25 ± 5	163 ± 15	25 ± 5	0
	13 ± 3%	87 ± 8%	100%	–
Jejunum MP	22 ± 6	221 ± 18	22 ± 6	0
	9 ± 2%	91 ± 7%	100%	–
Jejunum SMP	55 ± 7	77 ± 13	55 ± 7	0
	42 ± 5%	58 ± 10%	100%	–
Ileum MP	19 ± 4	167 ± 16	19 ± 4	0
	10 ± 2%	90 ± 9%	100%	–
Ileum SMP	63 ± 6	78 ± 8	63 ± 6	0
	45 ± 4%	55 ± 6%	100%	–
Distal colon MP	29 ± 6	234 ± 15	29 ± 6	0
	11 ± 2%	89 ± 6%	100%	–
Distal colon SMP	81 ± 9	87 ± 14	81 ± 9	0
	48 ± 5%	52 ± 8%	100%	–

The *first column* shows the mean number of $P2Y_2$-ir neurons also labeled with NPY ± SE mean, expressed as a percentage underneath. The *second column* shows the mean number of $P2Y_2$-ir neurons that were not immunopositive for NPY ± SE mean, expressed as a percentage underneath. The *third column* shows the mean number of NPY-ir neurons also immunopositive for $P2Y_2$ receptors ± SE mean, expressed as a percentage underneath. The *final column* shows the mean number of NPY-ir neurons that were not immunopositive for $P2Y_2$ receptors ± SE mean, expressed as a percentage underneath

Table 3 Quantitative analysis of double labeling studies between $P2Y_2$ receptors and calretinin (*CR*) in the myenteric plexus (*MP*) and submucous plexus (*SMP*) of guinea pig stomach corpus, jejunum, ileum and distal colon

Region	$P2Y_2$-ir + CR-ir +	$P2Y_2$-ir + CR-ir −	CR-ir + $P2Y_2$-ir +	CR-ir + $P2Y_2$-ir −
Stomach corpus MP	43 ± 8	151 ± 15	43 ± 8	105 ± 12
	22 ± 4%	78 ± 8%	29 ± 5%	71 ± 8%
Jejunum MP	199 ± 14	31 ± 7	199 ± 14	38 ± 6
	87 ± 6%	13 ± 3%	84 ± 6%	16 ± 3%
Jejunum SMP	56 ± 5	69 ± 12	56 ± 5	36 ± 6
	45 ± 4%	55 ± 9%	61 ± 5%	39 ± 6%
Ileum MP	224 ± 13	42 ± 6	224 ± 13	47 ± 5
	84 ± 5%	16 ± 2%	83 ± 5%	17 ± 2%
Ileum SMP	55 ± 7	63 ± 8	55 ± 7	32 ± 6
	47 ± 4%	53 ± 7%	63 ± 8%	37 ± 7%
Distal colon MP	95 ± 15	172 ± 21	95 ± 15	164 ± 19
	36 ± 6%	64 ± 8%	37 ± 6%	63 ± 7%
Distal colon SMP	59 ± 6	77 ± 11	59 ± 6	43 ± 5
	43 ± 4%	57 ± 8%	58 ± 6%	42 ± 5%

The *first column* shows the mean number of $P2Y_2$-ir neurons also labeled with CR ± SE mean, expressed as a percentage underneath. The *second column* shows the mean number of $P2Y_2$-ir neurons that were not immunopositive for CR ± SE mean, expressed as a percentage underneath. The *third column* shows the mean number of CR-ir neurons also immunopositive for $P2Y_2$ receptors ± SE mean, expressed as a percentage underneath. The *final column* shows the mean number of CR-ir neurons that were not immunopositive for $P2Y_2$ receptors ± SE mean, expressed as a percentage underneath

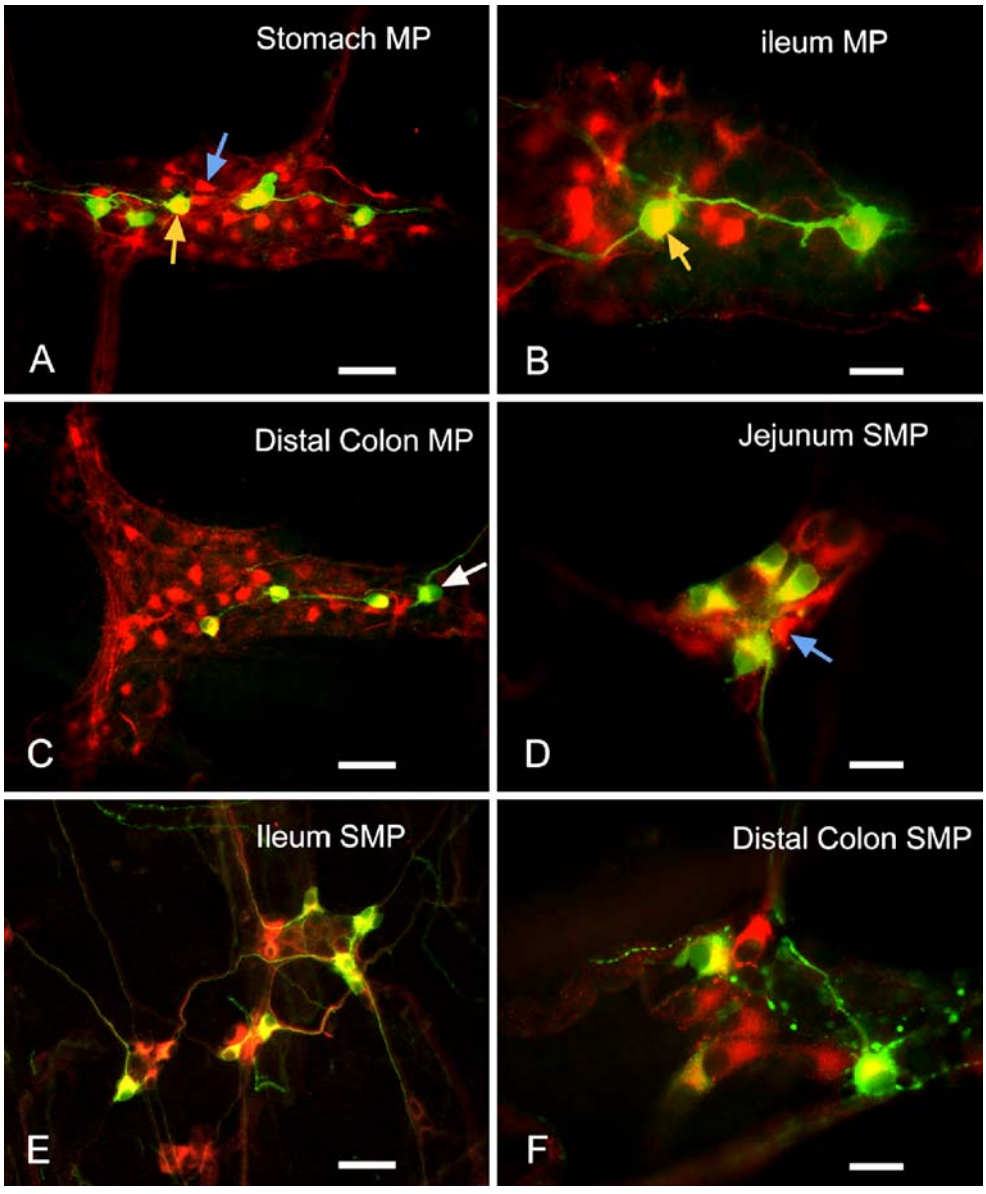

Fig. 2 Coexistence of $P2Y_2$-ir and NPY-ir in myenteric and submucosal plexuses of guinea pig stomach corpus, small intestine and distal colon. **a–c** show coexistence (*yellow*) between $P2Y_2$-ir (*red*) and NPY-ir (*green*) in myenteric plexus of stomach corpus, ileum and distal colon respectively. **d–f** show coexistence in submucous plexus of jejunum, ileum and distal colon, respectively. A *blue arrow* indicates a single neuron labelled with $P2Y_2$ antibody (*red*), a *white arrow* indicates a single neuron labeled with NPY antibody (*green*) and a *yellow arrow* indicates a neuron double labeled with both $P2Y_2$ and NPY antibodies (*yellow*). *Scale bars* in a–f = 50 μm

attached to a Zeiss Axioplan microscope (Zeiss, Germany). Filter sets included the following: for Cy3, excitation 510–550 nm, emission 590 nm; for FITC, 470 nm excitation, 525 nm emission. Images were imported into a graphics package (Adobe Photoshop 5.0, USA). The two-channel readings for green and red fluorescence were merged by using Adobe-Photoshop 5.0.

Quantitative analysis

Whole-mount preparations were used to perform a quantitative analysis, as described previously (Van Nassauw et al. 2002). Briefly, the immunoreactive-positive neurons in the myenteric ganglia were counted per visual field (0.3 mm^2) in whole-mount preparations. Ten randomly chosen fields in each whole-mount preparation were studied, and the number of immunoreactive neurons was calculated as a percentage of the total number of neurons as visualized with PGP9.5. A recent paper has provided evidence that the pan-neuronal markers Cuprolinic Blue and anti-HuC/D may be more reliable neuronal markers, visualising a greater number of neurons than PGP9.5, for use in future studies (Phillips et al. 2004).

Results

Localization of $P2Y_2$ receptor immunoreactivity

The $P2Y_2$ receptor immunoreactivity was found in the myenteric plexus of stomach corpus, jejunum, ileum and colon of the guinea pig (see Table 1), since the proximal

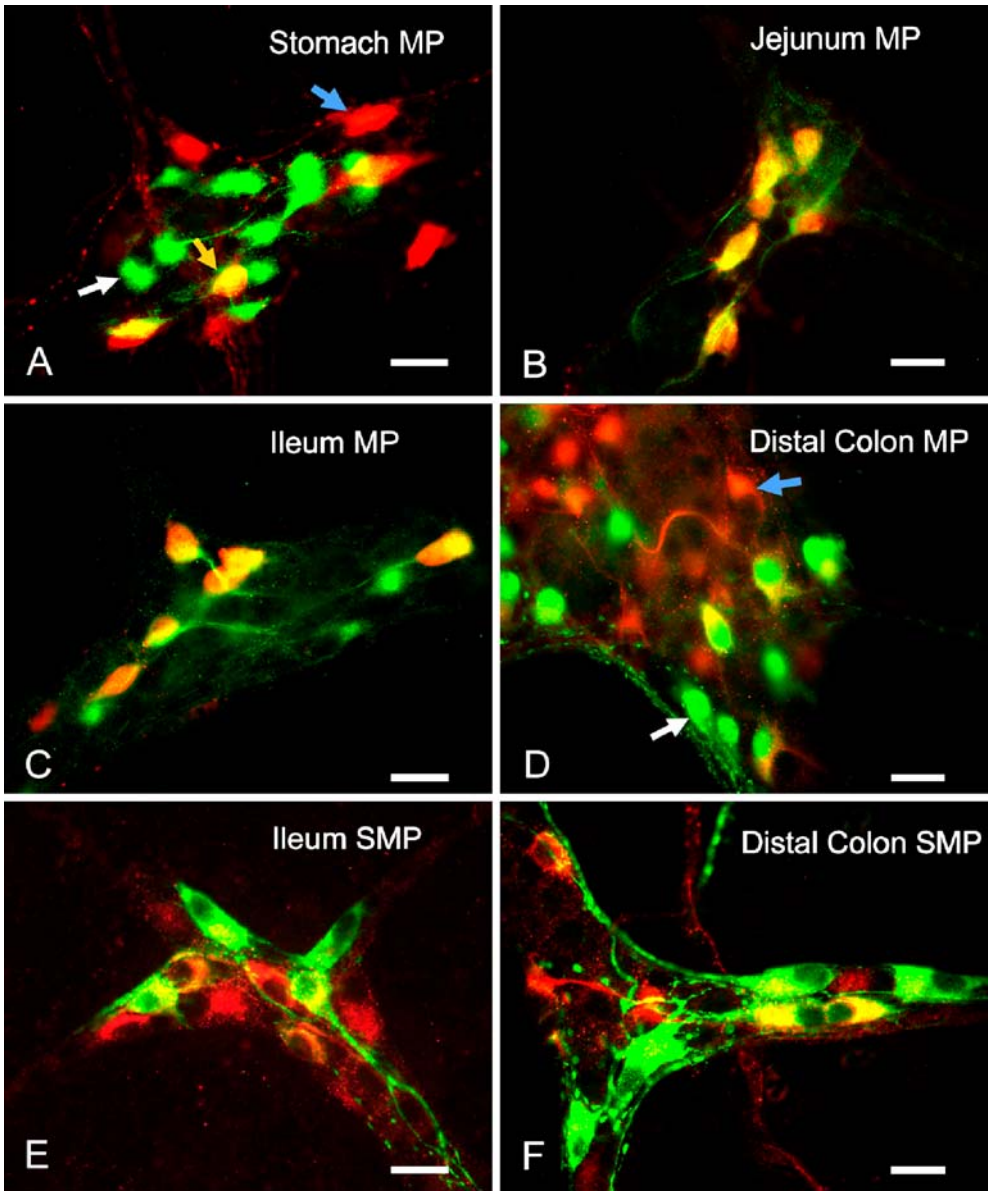

Fig. 3 Coexistence between $P2Y_2$-ir and calretinin (*CR*)-ir in myenteric and submucous plexuses in the guinea pig stomach corpus, small intestine and distal colon. **a**–**d** show coexistence between $P2Y_2$-ir and CR-ir in myenteric plexus of stomach corpus, jejunum, ileum and distal colon, respectively; a *blue arrow* indicates a single neuron labeled with $P2Y_2$ antibody (*red*), a *white arrow* indicates a single neuron labeled with CR antibody (*green*) and a *yellow arrow* indicates a neuron double labeled with both $P2Y_2$ and CR antibodies (*yellow*). **e** and **f** show coexistence between $P2Y_2$-ir and CR-ir in submucous plexus in ileum and distal colon. *Scale bars* in a–f = 50 μm

and distal colon were similar, only findings of the distal colon shall be presented. Most of the positive neurons were shown to have a long process with strong positive staining and most of the positive staining was usually seen on this side of the cell body, while on the other side the staining was much weaker. No staining was observed in the oval nucleus of the positive nerve cells. Two types of positive ganglion neurons, strongly staining and weakly staining nerve cells, were present in myenteric and submucousal plexuses. Most of the strongly stained nerve cells had a long process while the weakly stained nerve cell had no processes. (Fig. 1). In the stomach corpus myenteric plexus, approximately 38% of ganglion cells were positively stained with the $P2Y_2$ antiserum and two types of positive ganglion neurons, strongly staining and weakly staining nerve cells, were present (Fig. 1a). In the small intestine myenteric plexus of whole-mount preparations, $P2Y_2$-ir ganglion neurons were found in all ganglia and approximately 54 and 63% of ganglion cells were positively immunostained for the $P2Y_2$ antibody in the jejunum and ileum myenteric plexuses, respectively (Fig. 1b, c). In the myenteric plexus of distal colon, approximately 65% of ganglion neurons were immunostained intensely by the $P2Y_2$ antiserum (Fig. 1d, e). The $P2Y_2$-ir ganglion cells were also found in the submucosal plexus of those areas of the digestive tract examined. Two types of positive ganglion neurons, strongly staining and weakly staining nerve cells, were also present. Approximately 42, 46 and 45% of ganglion cells were $P2Y_2$-ir in the submucosal plexus of jejunum, ileum and distal colon, respectively (Fig. 1f–h).

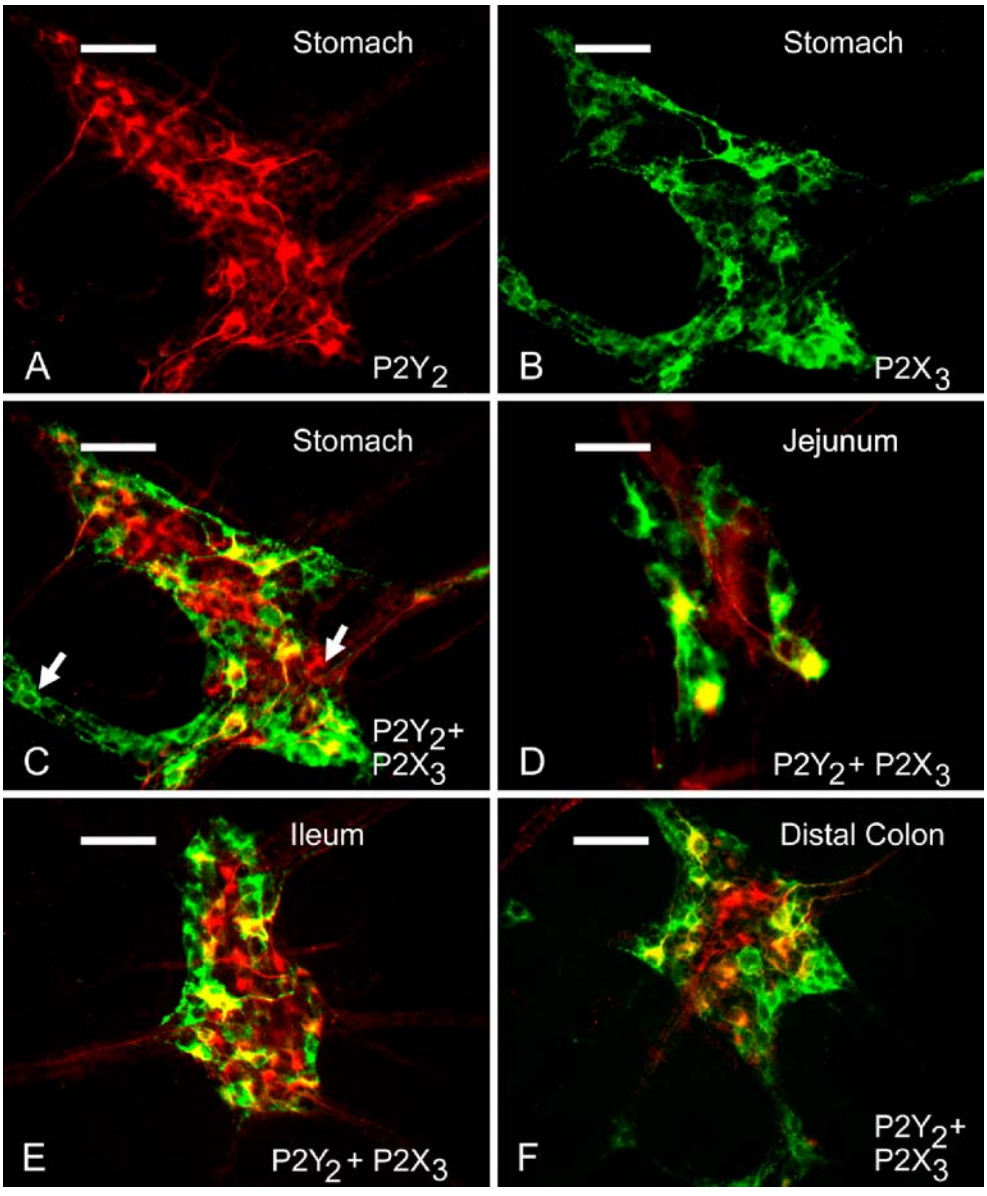

Fig. 4 Coexistence between $P2Y_2$-ir and $P2X_3$-ir in myenteric plexus in the guinea pig stomach corpus, small intestine and distal colon. **a** $P2Y_2$-ir neurons and fibers in myenteric plexus in stomach corpus (*red*). **b** $P2X_3$-ir neurons and fibers in myenteric plexus in stomach corpus (*green*). **c** The merged figure from a and b showing coexistence of $P2Y_2$ and $P2X_3$ receptors (*yellow*). **d**, **e** and **f** show coexistence (*yellow*) of $P2Y_2$-ir (*red*) and $P2X_3$-ir (*green*) in myenteric plexus of jejunum, ileum and distal colon, respectively. *Scale bars* in a–f = 50 μm

Double-labeling studies

The $P2Y_2$-ir was found to coexist with NPY, calretinin and $P2X_3$ receptors in myenteric plexus of stomach corpus, jejunum, ileum, and distal colon and in submucous plexus of jejunum, ileum, and distal colon.

All the ganglion cells with NPY-ir in both myenteric and submucosal plexuses were also immunoreactive for $P2Y_2$, although only 13, 9, 10 and 11% of the ganglion cells with $P2Y_2$-ir were immunoreactive for NPY in the myenteric plexuses of stomach corpus, jejunum, ileum and distal colon, respectively, and 42, 45 and 48% of the ganglion cells with $P2Y_2$-ir were immunoreactive for NPY in the submucousal plexuses of jejunum, ileum and distal colon, respectively (Fig. 2a–f). Table 2 shows the quantitative analysis of double labeling of $P2Y_2$ receptors with NPY in the myenteric and submucosal plexuses.

About 22, 87, 84 and 36% of $P2Y_2$-ir ganglion cells showed immunoreactivity for CR in the myenteric plexuses of the stomach corpus, jejunum, ileum and distal colon, and 45, 47 and 43% $P2Y_2$-ir ganglion cells showed immunoreactivity for CR in the myenteric plexuses of the jejunum, ileum and distal colon, respectively, although there were also CR-ir ganglion cells with no $P2Y_2$ immunoreactivity in both myenteric and submucosal plexuses (Fig. 3a–f). Table 3 shows the quantitative analysis of double labeling of $P2Y_2$ receptors with calretinin in myenteric and submucosal plexuses.

About 46, 52, 52 and 46% $P2X_3$-ir ganglion cells were found to display $P2Y_2$ receptor-ir in the myenteric plexuses of the stomach corpus, jejunum, ileum and distal colon, respectively. However, in the submucous plexus of the jejunum, ileum and distal colon all the $P2X_3$-ir ganglion cells were found to display $P2Y_2$ receptor-ir, but only 30, 37 and 33% of the ganglion cells

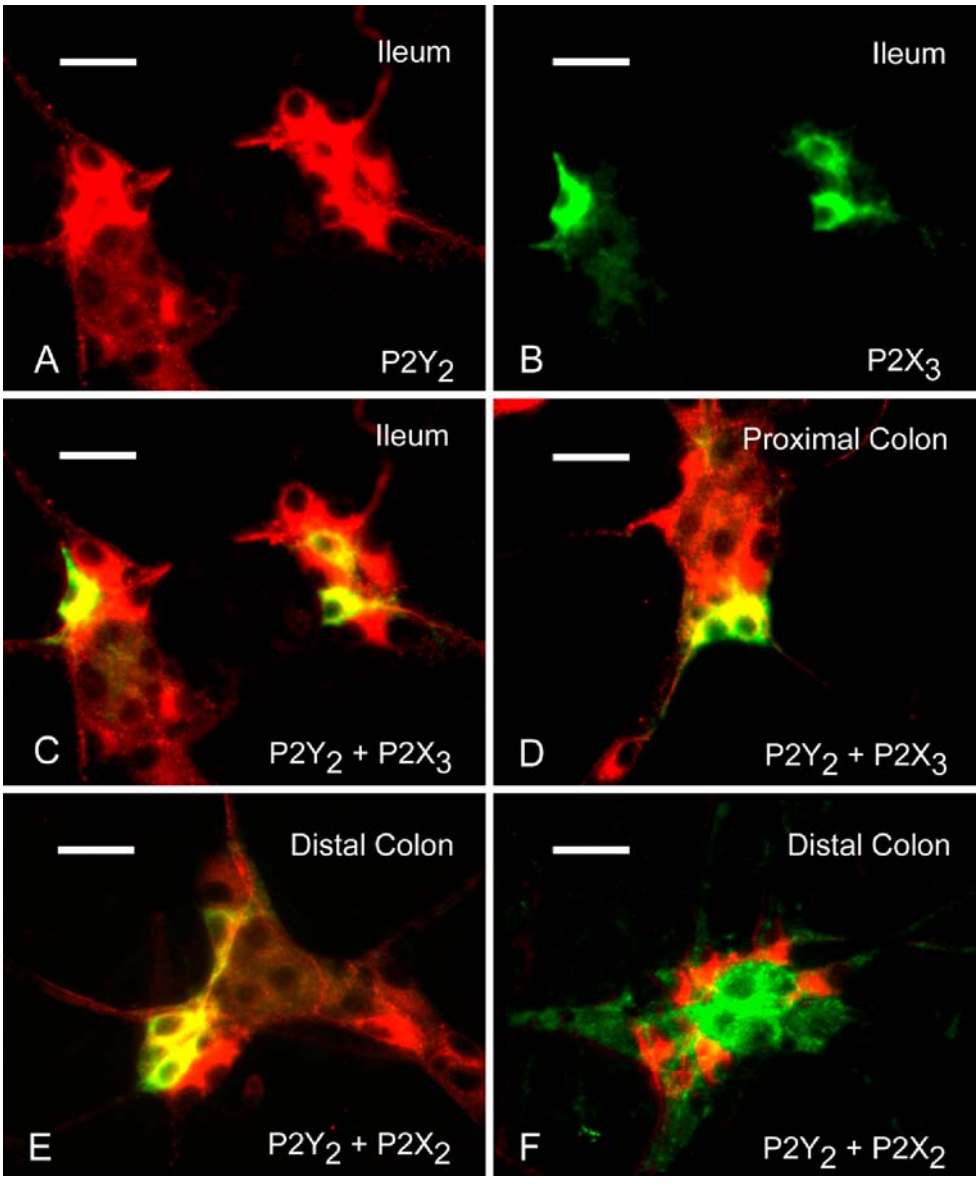

Fig. 5 Coexistence between $P2Y_2$-ir, $P2X_2$-ir and $P2X_3$-ir in submucous plexus in the guinea pig small intestine and distal colon. **a** $P2Y_2$-ir neurons in submucous plexus of ileum. **b** $P2X_3$-ir neurons in submucous plexus of ileum. **c** The merged figure from a and b showing coexistence of $P2Y_2$ and $P2X_3$ receptors (*yellow*). **d** and **e** show coexistence (*yellow*) between $P2Y_2$- (*red*) and $P2X_3$-ir (*green*) in submucous plexus of proximal and distal colon, respectively. **f** Co-localization between $P2Y_2$-ir (*red*) and $P2X_2$-ir (*green*) in submucous plexus of distal colon, note that there was no double-labeling in neurons and fibers. *Scale bars* in a-f = 50 μm

with $P2Y_2$-ir were immunoreactive for $P2X_3$ receptors (Figs. 4a–f, 5a–f). Table 4 shows the quantitative analysis of double labeling of $P2Y_2$ receptors with $P2X_3$ receptors in the myenteric and submucous plexuses.

No $P2Y_2$-ir ganglion cells were found to be immunoreactive for $P2X_2$ receptors, calbindin or NOS (Figs. 5a–f, 6f).

Discussion

The P2X receptors have been shown to play an important role in synaptic transmission within the neural pathways mediating motor activity in the intestine (Katayama and Morita 1989; Kimball et al. 1996; Heinemann et al. 1999; LePard and Galligan 1999; Bian et al. 2000; Galligan et al. 2000; Spencer et al. 2000; Ren et al. 2003) and the non-cholinergic portion of fast excitatory postsynaptic potentials are mediated by P2X receptors, with similarities to $P2X_4$ and $P2X_6$ receptor subunits in the submucous and myenteric plexuses (Barajas-Lopez et al. 1994, 2000; Galligan and Bertrand 1994; LePard et al. 1997; Zhou and Galligan 1998; Burnstock 2001). In addition to fast responses, ATP activates a P2Y receptor subunit to cause a slow membrane depolarization in a subset of S/type 1 neurons of the submucous plexus of the guinea pig ileum (Barajas-Lopez et al. 1994, 2000; Hu et al. 2003). Not all S/type 1 neurons have both fast and slow membrane depolarization responses. Three subsets of cells could be distinguished: those with both fast and slow responses, those with only fast responses and those with only slow responses (Barajas-Lopez et al. 2000). These results suggested that a subset of S/type 1 neurons exist in the submucous plexus of the guinea pig that express both P2X and P2Y receptors. In this study, we used a double-labeling immunofluorescence technique to demonstrate a subset of neurons expressing both $P2X_3$ and $P2Y_2$

Table 4 Quantitative analysis of double labeling studies between $P2Y_2$ and $P2X_3$ receptors in the myenteric plexus (*MP*) and submucous plexus (*SMP*) of guinea pig stomach corpus, jejunum, ileum and distal colon

Region	$P2Y_2$-ir + $P2X_3$-ir +	$P2Y_2$-ir + $P2X_3$-ir −	$P2X_3$-ir + $P2Y_2$-ir +	$P2X_3$-ir + $P2Y_2$-ir −
Stomach corpus MP	107 ± 21	85 ± 15	107 ± 21	135 ± 28
	56 ± 11%	44 ± 8%	44 ± 6%	56 ± 9%
Jejunum MP	155 ± 28	98 ± 12	155 ± 28	138 ± 17
	61 ± 12%	39 ± 8%	52 ± 10%	48 ± 8%
Jejunum SMP	37 ± 7	87 ± 14	37 ± 7	0
	30 ± 5%	70 ± 9%	100%	–
Ileum MP	169 ± 25	122 ± 14	169 ± 25	177 ± 29
	58 ± 16%	42 ± 9%	52 ± 15%	48 ± 12%
Ileum SMP	48 ± 7	83 ± 9	48 ± 7	0
	37 ± 5%	63 ± 8%	100%	–
Distal colon MP	178 ± 28	105 ± 15	178 ± 28	207 ± 26
	63 ± 7%	37 ± 5%	46 ± 11%	54 ± 13%
Distal colon SMP	51 ± 9	104 ± 14	51 ± 9	0
	33 ± 6%	67 ± 8%	100%	–

The *first column* shows the mean number of $P2Y_2$-ir neurons also labeled with $P2X_3$ receptor antibodies ± SE mean, expressed as a percentage underneath. The *second column* shows the mean number of $P2Y_2$-ir neurons that were not immunopositive for $P2X_3$ receptors ± SE mean, expressed as a percentage underneath. The *third column* shows the mean number of $P2X_3$-ir neurons also immunopositive for $P2Y_2$ receptors ± SE mean, expressed as a percentage underneath. The *final column* shows the mean number of $P2X_3$-ir neurons that were not immunopositive for $P2Y_2$ receptors ± SE mean, expressed as a percentage underneath

receptors in myenteric and submucousal plexuses of stomach corpus, jejunum, ileum and distal colon. This subset of neurons could be the candidate for this functional subset of S/type 1. Furthermore, the subunit of P2X receptor that coexists with $P2Y_2$ receptors is of the $P2X_3$, but not the $P2X_2$ receptor subunit. In the present study, all the $P2X_3$-ir neurons were found to also express $P2Y_2$ receptors in submucous plexus neurons. So there must be at least one other P2X receptor subunit in the submucosal plexus since three functional subsets of neurons have been distinguished (Barajas-Lopez et al. 2000). In fact, other subunits of P2X receptors ($P2X_2$ and $P2X_7$) have been identified in submucous plexus by immunofluorescence (Hu et al. 2001; Castelucci et al. 2002, 2003; Xiang and Burnstock 2004b).

The shape of all the $P2Y_2$-ir neurons demonstrated in this study in both myenteric and submucosal plexuses of all the regions of guinea pig gut that we examined was characteristic, with only one axon-like process present. This result suggests that most of $P2Y_2$-ir neurons in guinea pig gut have Dogiel type 1 neuron morphology. Most of the Dogiel type 1 neurons are S neurons with electrophysiological properties of monophasic depolarizations, no slow after hyperpolarization, and fast excitatory postsynaptic potentials in response to fiber tract stimulation (Nurgali et al. 2004). Our results together with those of Nurgali et al. (2004) imply that some $P2Y_2$-ir neurons in both myenteric and submucosal plexuses in all regions of guinea pig gut are likely to be S/Dogiel type 1 neurons.

The $P2Y_2$-ir neurons in the myenteric and submucosal plexuses are not intrinsic primary afferent neurons as they do not express calbindin, which is believed to be a marker for intrinsic sensory neurons in the guinea pig intestine (Furness et al. 1990; Quinson et al. 2001). The morphology of intrinsic sensory neurons in the guinea pig ileum is distinct; their shape and projection patterns fit with those of Dogiel type-2 neurons (Furness et al. 1998).

The $P2Y_2$-ir neurons were found not to express NOS-ir, although NOS-ir neurons have been found to label S/type 1 neurons in guinea pig myenteric plexus exhibiting uniaxonal and Dogiel type 1 morphology (McConalogue and Furness 1993). These data imply that a subpopulation of S/type 1 neurons in the myenteric and submucosal plexuses of guinea pig do not express $P2Y_2$-ir.

In this study, we observed that there were obvious regional differences in the percentages of coexistence of $P2Y_2$ receptors with CR, NPY and $P2X_3$ receptors. For example, 84–87% $P2Y_2$ receptor-ir neurons were also immunoreactive for CR in the myenteric plexus of the small intestine while only 36% were CR-ir in the distal colon. In the myenteric plexus only 9–11% $P2Y_2$ receptor-ir neurons were found to express NPY-ir while in the submucousal plexus almost half the $P2Y_2$ receptor-ir neurons were also immunoreactive for NPY. Since the function of the digestive tract varies with region, so too does the morphology and the neurochemical coding of their nerves (Furness 2000). Such regional differences have been observed in this study. The density of NPY-ir neuronal cell bodies and fibers in the submucosal plexus was high in the ascending colon and progressively declined in an anal direction, immunoreactive cell bodies being rare in the rectum. The potentially important regional differences in the functions of neuropeptide Y as an antisecretory peptide in the local regulation of chloride transport in the mucosa and as a modulator of ganglionic transmission has been proposed (Cunningham and Lees 1995).

In the myenteric and submucosal plexuses of the guinea pig gastrointestinal tract, especially, the small intestine, the majority of the $P2Y_2$-ir neurons were also immunoreactive for calretinin in this study. Calretinin is believed to be a marker for cholinergic neurons in the guinea pig intestine, being Dogiel type 1 neurons, which project to the longitudinal muscle, the submucosal vasculature and mucosal glands (Brookes et al. 1991) and control the physiological functions of these structures. Thus, extracellular ATP may regulate the function of these cholinergic neurons and thereby indirectly regulate the physiological functions of smooth muscle, submucosal blood vessels and mucosal glands.

In the present study almost all the NPY-ir neurons were also immunoreactive for $P2Y_2$ receptors. The NPY-ir neurons were localized in myenteric and submucosal plexuses, which project to the circular muscle, the longitudinal muscle and the mucosa; these NPY-ir neurons also show Dogiel type 1 neuron morphology,

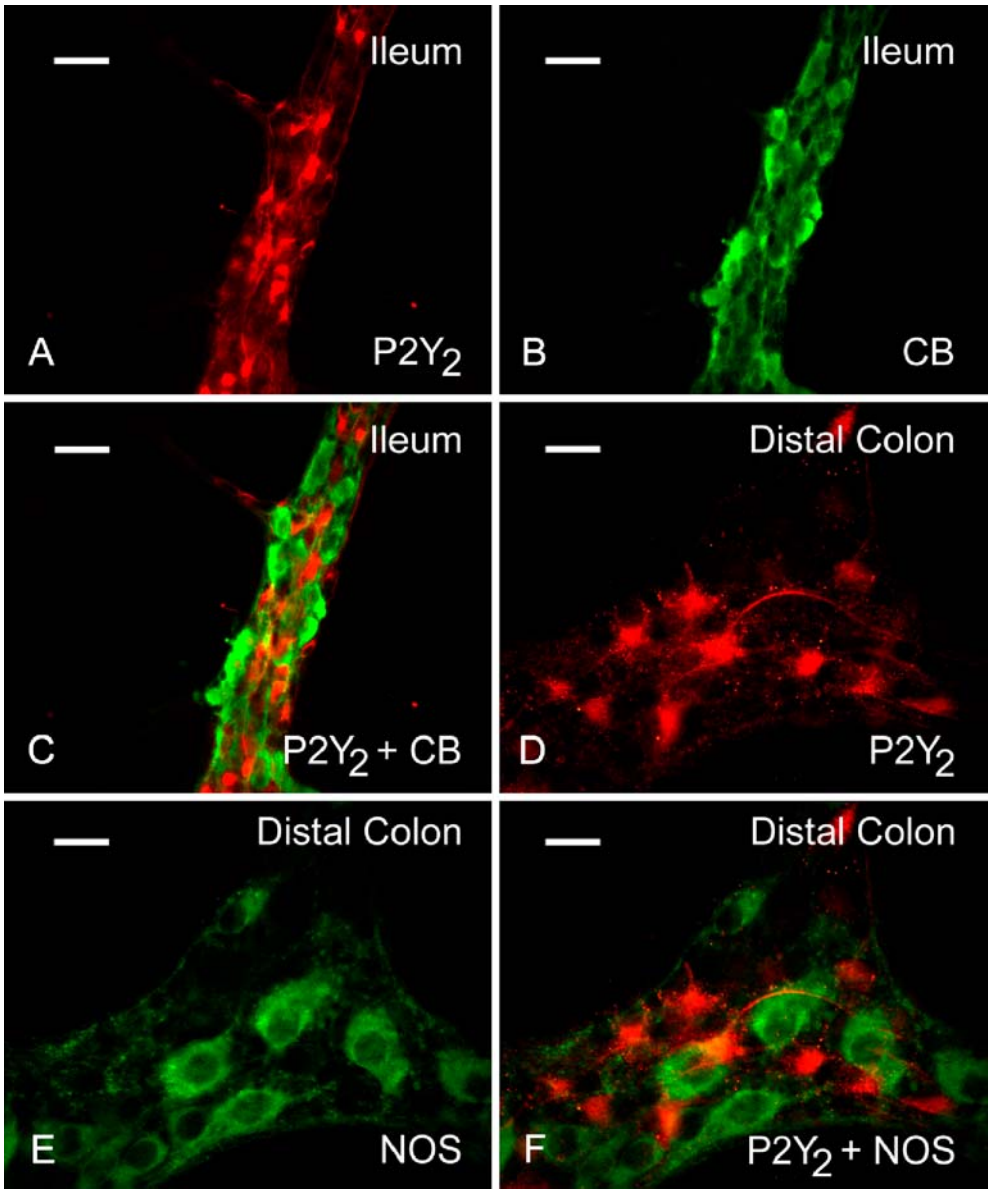

Fig. 6 Co-localizations among $P2Y_2$-ir, calbindin (*CB*) and NOS in myenteric and submucous plexuses in the ileum and distal colon. **a** $P2Y_2$-ir neurons and fibers in myenteric plexus of ileum. **b** CB-ir neurons and fibers in myenteric plexus of guinea pig ileum. **c** The merged figure from a and b, note that there was no double-labeling. **d** $P2Y_2$-ir neurons in myenteric plexus of distal colon. **e** NOS-ir neurons in myenteric plexus of distal colon. **f** The merged figure from d and e; note that there was no double-labeling. *Scale bars* in a-f = 50 μm

except those projecting to mucosa which have fine branching processes (Uemura et al. 1995). These data imply that the physiological function of NPY-ir neurons in myenteric and submucosal plexuses can be regulated by extracellular ATP.

In summary, P2Y-ir neurons and fibers were found to be distributed widely in myenteric and submucosal plexuses in the guinea pig stomach corpus, jejunum, ileum and colon. The typical morphology of P2Y-ir neuron is that they have one long process and the distribution of positive staining in the cell body is polarized. The P2Y-ir neurons are Dogiel type 1. Double-labeling studies showed that 40–60% of $P2X_3$-ir neurons were immunoreactive for $P2Y_2$ receptors in the myenteric plexus and all the $P2Y_2$-ir neurons were immunoreactive for $P2X_3$ receptors in the submucosal plexus; almost all the NPY-ir neurons and the majority of CR-ir neurons were also immunoreactive for $P2Y_2$ receptors. However, no $P2Y_2$-ir neurons were immunoreactive for $P2X_2$ receptors, calbindin or NOS. It is shown for the first time that S/Dogiel type 1 neurons with fast P2X and slow P2Y receptor-mediated depolarizations are those neurons, which express both $P2Y_2$-ir and $P2X_3$-ir and that they are widely distributed in myenteric and submucosal plexuses of guinea pig gut. This appears to be in contrast to mouse intestine where $P2X_3$ receptor knockout studies suggest that $P2X_3$ subunits are localised on AH (sensory), but not S neurons (Bian et al. 2003).

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