Histochem Cell Biol (2002) 118:193–203
DOI 10.1007/s00418-002-0447-6

ORIGINAL PAPER

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Neurochemical identification of enteric neurons expressing $P2X_3$ receptors in the guinea-pig ileum

Accepted: 21 June 2002 / Published online: 23 July 2002

Abstract It was hypothesised that $P2X_3$ receptors, predominantly labelling spinal and cranial sensory ganglionic neurons, are also expressed in intrinsic sensory enteric neurons, although direct evidence is lacking. The aim of this study was to localise $P2X_3$ receptors in the enteric nervous system of the guinea-pig ileum, and to neurochemically identify the $P2X_3$-expressing neurons. In the submucous plexus, cholinergic neurons expressing calretinin (CRT), were immunostained for $P2X_3$. These neurons made up about 12% of the submucous neurons. In the myenteric plexus, approximately 36% of the neurons expressed $P2X_3$. Half of the latter neurons were immunoreactive for CRT, whereas about 20% were immunoreactive for nitric oxide synthase (NOS). Based on earlier neurochemical analysis of enteric neurons in the guinea-pig, the myenteric neurons exhibiting $P2X_3$/CRT immunoreactivity were identified as longitudinal muscle motor neurons, and those expressing $P2X_3$/NOS immunoreactivity as short inhibitory circular muscle motor neurons. In both plexuses, no colocalisation was observed between $P2X_3$ and calbindin, a marker for intrinsic sensory neurons. Multiple staining with antisera raised against somatostatin, neuropeptide Y, substance P or neurofilament protein did not reveal any costaining. It can be concluded that in the guinea-pig ileum, intrinsic sensory neurons do not express $P2X_3$ receptors. However, this does not negate the possibility that extrinsic sensory nerves expressing $P2X_3$ are involved in a purinergic mechanosensory transduction pathway as demonstrated in other organs.

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Keywords $P2X_3$ receptor · Ileum · Enteric nervous system · Guinea-pig · Sensory neurons

Introduction

Although this topic has been a subject of much dispute for several decades, there is nowadays overwhelming evidence that purinergic signalling is widespread. Two families of purinoceptors have been identified, a P2X ionotropic ligand-gated ion channel family and a P2Y metabotropic G protein-coupled family. To date, seven mammalian P2X receptor subunits ($P2X_1$–$P2X_7$), which can assemble to form homomeric and heteromeric receptors, have been identified by molecular cloning (for review see Burnstock 2001a; Dunn et al. 2001; Khakh et al. 2001). The $P2X_3$ receptors have attracted special attention in that they predominantly label sensory neurons, particularly the small nociceptive neurons in the dorsal root, trigeminal and nodose ganglia (Vulchanova et al. 1996, 1997; Cook et al. 1997; Bradbury et al. 1998; Llewellyn-Smith and Burnstock 1998; Burnstock 2000; Hubscher et al. 2001).

ATP has been shown to elicit responses of neurons in both plexuses of the enteric nervous system (ENS), indicating the presence of P2X receptors (for review see Burnstock 2001b; Dunn et al. 2001). There has been some ambiguity about the interpretation of the electrophysiological results regarding which subtypes of P2X receptors are present in enteric neurons. It has been suggested that enteric neurons express predominantly $P2X_2$ receptors (Zhou and Galligan 1996; LePard et al. 1997), while a minority heterologously expresses $P2X_1$ and $P2X_3$ receptors (Zhou and Galligan 1996). Barajas-López et al. (1996) have observed that myenteric neurons express a P2X receptor showing some pharmacological resemblance to $P2X_4$ and $P2X_6$ receptors. Moreover, they have noted that the electrophysiological and pharmacological properties of P2X receptors are virtually identical in both myenteric and submucous neurons, suggesting that similar P2X receptors are present in both plexuses.

To date, few morphological data are available on the presence of P2X immunoreactivity (IR) in the ENS. Most data concern the presence of $P2X_2$ IR in the guinea-pig ENS (Vulchanova et al. 1996; Hu et al. 2001; Castelucci et al. 2002). The $P2X_2$ receptor has been found to be expressed by specific subtypes of enteric neurons, including inhibitory motor neurons, non-cholinergic secretomotor neurons and intrinsic primary afferent neurons (Castelucci et al. 2002). $P2X_3$ IR has been detected in some submucous and myenteric neurons of the human colon (Yiangou et al. 2000, 2001; Facer et al. 2001), while $P2X_7$ IR has been found in nerve fibres and somata in both myenteric and submucous plexuses of the guinea-pig small intestine (Hu et al. 2001). In addition, it has been suggested that intrinsic sensory neurons in both ganglionic plexuses express IR for the $P2X_3$ receptor, implicating a possible role of these neurons in reflex pathways (Burnstock 2001c).

Distension of bowel segments may evoke non-painful and painful sensations, regulating reflexes (Jänig and Koltzenburg 1988). Both intrinsic (Furness et al. 1988; Song et al. 1991, 1992, 1994; Kirchgessner et al. 1992; Neunlist and Schemann 1997; Meedeniya et al. 1998; Neunlist et al. 1999; Hens et al. 2000, 2001) and extrinsic (for review see Grundy and Scratcherd 1989; Cervero 1994; Berthoud and Neuhuber 2000) sensory neurons project to the mucosa. It has recently been proposed that ATP released from mucosal epithelial cells during moderate distension of the intestine acts on $P2X_3$ receptors present on subepithelial sensory processes of intrinsic enteric neurons to trigger peristalsis, while severe distension acts on $P2X_3$ receptors on subepithelial sensory processes of extrinsic sensory neurons originating in the dorsal root ganglia to relay to pain centres in the brain (Burnstock 2001c). In view of the putative role of $P2X_3$ receptors in this hypothesis of purine-mediated mechanosensory transduction in the gut, the aim of the present study was to immunocytochemically localise the $P2X_3$ receptor in the ENS of the guinea-pig ileum, and, based on our current extensive knowledge of their neurochemical coding (Costa et al. 1996; Brookes 2001), to identify the functional subpopulations of enteric neurons expressing $P2X_3$ receptors.

Materials and methods

Tissue preparation

Tissue was obtained from adult guinea-pigs (Iffa Credo Belgium, Brussels, Belgium), weighing ca 350 g, of both genders, which were stunned by a blow to the head and killed by severing the carotid arteries and spinal cord. All procedures were approved by the local ethics committee of the University of Antwerp. Segments of the ileum were removed and rinsed in Krebs solution (117 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2 \cdot 2H_2O$, 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 ileum, a 5-mm segment was removed and processed for cryosectioning (cfr. infra). The remaining part of each ileum was opened along the mesenteric border and pinned out in a Sylgard-lined Petri dish filled with fixative (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.0) 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 optimise the immunocytochemical staining as previously described (Llewellyn-Smith et al. 1985). To enhance the IR for neuropeptides within the neuronal somata, some preparations were, prior to fixation, maintained in organotypic culture in sterile Dulbecco's modified Eagle medium (Sigma, St. Louis, Mo., USA) supplemented with 10 mg/ml antibiotic-antimycotic (Sigma), 50 µg/ml gentamycin (Sigma), 2.5 µg/ml amphotericin B (Sigma), 10% fetal bovine serum (Sigma), 1 µM nifedipine, 2.1 mg/ml $NaHCO_3$ and 88 µM colchicine (Sigma), in a humidified CO_2 incubator (37°C, 5% CO_2) for 24 h, as previously described (Furness et al. 1989).

Following fixation and clearing, the tissues were dissected into two layers: the outer musculature with adhering serosa and the submucosa/mucosa. The circular muscle was removed to yield whole mounts of longitudinal muscle with the myenteric plexus attached. In a few whole mounts, the circular muscle was only partially removed. In the whole mounts containing the submucous layer, the mucosa was scraped off using a blunt scalpel. Square flat sheets of these mucosal parts were used for the examination of the subepithelial innervation. The whole mounts were stored in PBS containing 0.1% sodium azide.

The distal segment of each ileum was processed for cryosectioning. First, these segments were immersed in the above-mentioned fixative for 3 h at room temperature. They were rinsed in PBS, transferred to PBS-containing 20% sucrose and stored at 4°C overnight. Next, they were embedded in OCT embedding medium, sectioned in a Microm cryostat at 10 µm and thaw-mounted on gelatine-coated slides.

Primary antibody directed against $P2X_3$ receptor

The antibody directed against the $P2X_3$ receptor, raised by Roche Bioscience (Palo Alto, Calif., USA), has been detailed previously (Oglesby et al. 1999). The immunogen was a synthetic peptide representing 15 receptor-type-specific amino acids of the C-terminal part of the $P2X_3$ receptor (amino acids 383–397: VEKQSTDSGAYSIGH). This synthetic peptide was covalently linked to keyhole limpet haemocyanin. Rabbits were immunised with the conjugated peptide in multiple monthly injections (performed by Research Genetics, Huntsville, Ala., USA).

Immunoglobulin G (IgG) fractions were isolated from the preimmune and immune serum, according to the method of Harboe and Ingild (1973). The protein concentration was determined at 280 nm using an extinction factor of 1.43 for 1 mg/ml.

The specificity of the $P2X_3$ antibody was verified by immunoblotting with membrane preparations from cloned $P2X_{1-7}$ receptor expressing CHO-K1 cells. The antibody recognised only one protein of the expected size in the heterologous expression system and has been shown to be receptor subtype specific (Oglesby et al. 1999). Preabsorption of the antibody with excess of the synthetic peptide used for generation of the antibody, eliminated IR (Xiang et al. 1998a, b; Gröschel-Stewart et al. 1999).

Immunocytochemistry

All immunocytochemical incubations were carried out at room temperature. Unless indicated otherwise, washes with PBS were performed between each incubation. The antisera and streptavidin complexes used in this study as well as their respective dilutions, are listed in Table 1. Based on the extensive knowledge of the chemical coding of the different classes of enteric neurons in the guinea-pig (Costa et al. 1996; Brookes 2001), we have applied some of these neurochemical markers to further identify the $P2X_3$-immunoreactive enteric neurons in the present study (Table 2).

For detection of the $P2X_3$ receptor, the biotin-conjugated tyramide signal amplification (TSA) technique was applied, albeit slightly modified for whole-mount preparations (Brouns et al. 2000). Briefly, to minimise endogenous peroxidase activity, the

Table 1 List of antisera used for immunocytochemistry

Antigen	Host	Dilution	Source
Primary antisera			
$P2X_3$ receptor	Rabbit	1:500–1:1,000	Gift from Roche Bioscience, Palo Alto, Calif., USA (Oglesby et al. 1999)
c-Kit (C-19)	Rabbit	1:200	Santa Cruz Biotechnology, Santa Cruz, Calif., USA (sc-168)
Calbindin D-28 k	Rabbit	1:4,000	SWant, Bellinzola, Switzerland (CB38)
Calretinin	Rabbit	1:2,000	SWant
Neurofilament protein (iC8)	Mouse	1:500	Prof. C. Triban
Neuronal nitric oxide synthase	Rabbit	1:1,000	Euro-Diagnostica, Malmö, Sweden (B 220-1)
Neuronal nitric oxide synthase	Mouse	1:1,000	Sigma, St. Louis, Mo., USA (N2280)
Neuropeptide Y	Rabbit	1:100	Affiniti Research Products, Exeter, UK (NA1233)
Neuropeptide Y	Rat	1:100	Affiniti Research Products (NZ1115)
Somatostatin	Rabbit	1:100	Euro-Diagnostica (2263PSO)
Somatostatin	Rat	1:100	Biogenesis, Poole, UK (8330-0009)
Substance P	Rat	1:100	Biogenesis (8450-0505)
S100	Rabbit	1:1,000	Dako, Glostrup, Denmark (Z0311)
Secondary antisera and streptavidin complexes			
FITC-conjugated goat anti-mouse IgG		1:200	Jackson Immunoresearch Laboratories, West Grove, Pa., USA
FITC-conjugated goat anti-rat IgG		1:200	Jackson Immunoresearch Laboratories
Cy3-conjugated goat anti-rabbit IgG		1:4,000	Jackson Immunoresearch Laboratories
Cy5-conjugated goat anti-rabbit IgG		1:1,000	Jackson Immunoresearch Laboratories
FITC-conjugated Fab-fragments of goat anti-rabbit IgG		1:100	Jackson Immunoresearch Laboratories
Unlabelled Fab-fragments of goat anti-rabbit IgG		1:100	Jackson Immunoresearch Laboratories
Biotinylated Fab-fragments of goat anti-rabbit IgG		1:2,000	Rockland, Gilbertsville, PA
Cy3-conjugated streptavidin		1:4,000	Jackson Immunoresearch Laboratories
FITC-conjugated streptavidin		1:1,000	Jackson Immunoresearch Laboratories
ExtrAvidin-horseradish peroxidase		1:1,000	Sigma
Biotin-conjugated tyramide signal amplification kit			Perkin Elmer Life Sciences, Boston, Mass., USA

Table 2 Summary indicating which neuronal cell types are predominantly labelled by the used antisera and the possible colocalisation of the used markers. (*CB* Calbindin, *CM* circular muscle, *CRT* calretinin, *LM* longitudinal muscle, *MP* myenteric plexus, *NFP* neurofilament protein, *NOS* nitric oxide synthase, *NPY* neuropeptide Y, *SMP* submucous plexus, *SOM* somatostatin, *SP* substance P)

Antisera raised against	Neuronal cell types	Colocalisation with
CB	Primary afferent neuron (SMP)	SP[a]
	Primary afferent neuron (MP)	SP[a]
CRT	Vasodilator neuron (SMP)	–
	Excitatory motor neuron (MP)	SP[a]
	Ascending interneuron (MP)	NFP, SP
NOS	Inhibitory motor neuron, LM (MP)	NPY[a]
	Short inhibitory motor neuron, CM (MP)	–
	Long inhibitory motor neuron, CM (MP)	NFP
	Descending interneuron (MP)	NPY[a], NFP
NFP	Long inhibitory motor neuron, CM (MP)	NOS
	Ascending interneuron (MP)	CRT, SP
	Descending interneuron (MP)	NPY[a], NOS
	Descending interneuron (MP)	–
NPY	Secretomotor neuron (SMP)	SOM
	Viscerofugal neuron (MP)	SOM
SOM	Secretomotor neuron (SMP)	NPY
	Viscerofugal neuron (MP)	NPY
	Descending interneuron (MP)	–
SP	Ascending interneuron (MP)	CRT, NFP

[a] Some neurons of this neuronal cell type can display this marker

cryosections and whole mounts were treated with 3% hydrogen peroxide in methanol. Next, they were rinsed in PBS, and to eliminate endogenous avidin/biotin activity, the cryosections were treated with a blocking kit of Zymed Laboratories (San Francisco, Calif., USA). Subsequently, cryosections and whole-mount preparations were immersed in PBS containing 10% normal goat serum (NGS), 0.05% thimerosal and 1% Triton X-100, before incubation for 18 h (cryosections) or 48 h (whole mounts) with a polyclonal rabbit antibody directed against the $P2X_3$ receptor (cryosections: 1:1,000 dilution; whole mounts: 1:500 dilution) diluted in PBS containing 10% NGS, 0.05% thimerosal and 0.1% Triton X-100. The cryosections and whole mounts were consecutively incubated with biotinylated Fab fragments of goat anti-rabbit IgG diluted in PBS containing 1% NGS and 0.05% thimerosal, and with ExtrAvidin-horseradish peroxidase diluted in PBS containing 0.05% thimerosal and 0.1% bovine serum albumin (PBS+). Between subsequent steps, the cryosections and whole mounts were washed in PBS containing 0.05% Tween 20. Whole mounts and sections

Table 3 Results of the quantitative analysis of whole-mount preparations. *n*=number of whole mounts. (*IR* Immunoreactivity)

	Total number	Mean number/ whole-mount
Submucous plexus: double staining P_2X_3/CRT (*n*=5)		
Neurons expressing $P2X_3$ IR	247	49±4
Neurons expressing CRT IR	242	48±4
Neurons expressing $P2X_3$/CRT	236	47±4
Neurons expressing $P2X_3$/-	11	2±1
Neurons expressing -/CRT	6	1±2
Mean percentage of $P2X_3$-immunoreactive neurons expressing CRT IR/whole mount		95±2%
Mean percentage of CRT-immunoreactive neurons expressing $P2X_3$ IR/whole mount		97±3%
Myenteric plexus: double staining P_2X_3/CRT (*n*=5)		
Neurons expressing $P2X_3$ IR	845	169±32
Neurons expressing CRT IR	536	107±13
Neurons expressing $P2X_3$/CRT	417	83±14
Neurons expressing $P2X_3$/-	428	86±26
Neurons expressing -/CRT	119	24±7
Mean percentage of $P2X_3$-immunoreactive neurons expressing CRT IR/whole mount		50±7%
Mean percentage of CRT-immunoreactive neurons expressing $P2X_3$ IR/whole mount		78±7%
Myenteric plexus: double staining P_2X_3/NOS *n*=5)		
Neurons expressing $P2X_3$ IR	632	126±10
Neurons expressing NOS IR	533	107±17
Neurons expressing $P2X_3$/NOS	118	24±7
Neurons expressing $P2X_3$/-	514	103±10
Neurons expressing -/NOS	415	83±21
Mean percentage of $P2X_3$-immunoreactive neurons expressing NOS IR/whole mount		19±5%
Mean percentage of NOS-immunoreactive neurons expressing $P2X_3$ IR/whole mount		23±9%

were then incubated for 20 min in biotin-conjugated tyramide diluted in an amplification solution. Visualisation was performed using fluorophore-conjugated streptavidin diluted in PBS+. After washing, they were mounted in Citifluor. In double- and triple-labelling experiments, the $P2X_3$ receptor was always localised using the TSA method.

Double- and triple-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 technique was performed according to the method of Shindler and Roth (1996). In the first step, the TSA method was used to detect $P2X_3$ receptor, while in the second step a conventional immunofluorescence method was applied. For triple-labelling experiments using primary antisera raised in the same species, we performed a reliable sequential immunofluorescence labelling procedure developed by Brouns et al. (2002). Briefly, the first antigen, the $P2X_3$ receptor, was detected with the TSA method. The second antigen was visualised by means of a fluorophore-conjugated secondary monovalent Fab antibody. After incubation with unlabelled Fab fragments of goat anti-rabbit IgG, the third antigen was localised with a conventional immunofluorescence technique. In the sequential stainings, the primary antisera of the second and third step were diluted in PBS containing 10% NGS and 0.05% thimerosal. Secondary antisera were diluted in PBS containing 1% NGS and 0.05% thimerosal, while streptavidin complexes were diluted in PBS+.

Negative controls in which one of the primary antibodies was omitted, and interference control stainings were performed as described by Shindler and Roth (1996) and Brouns et al. (2002). The whole-mount preparations were evaluated with fluorescence and confocal microscopy.

The whole-mount preparations were also used to perform a quantitative analysis, as previously described (Van Nassauw et al. 2001). Briefly, the immunoreactive nerve cell bodies in the submucous and myenteric ganglia were counted per visual field (0.3 mm^2) in whole-mount preparations. Ten randomly chosen fields in each whole mount were analysed, and the number of immunoreactive neurons was calculated.

Results

Colchicine pretreatment enhanced immunostaining for the $P2X_3$ receptor within the guinea-pig ENS. Without pretreatment, IR was mainly limited to the soma, whereas with pretreatment, the proximal parts of the neuronal processes were also immunostained. Neurons containing $P2X_3$ receptors were localised in both the myenteric and

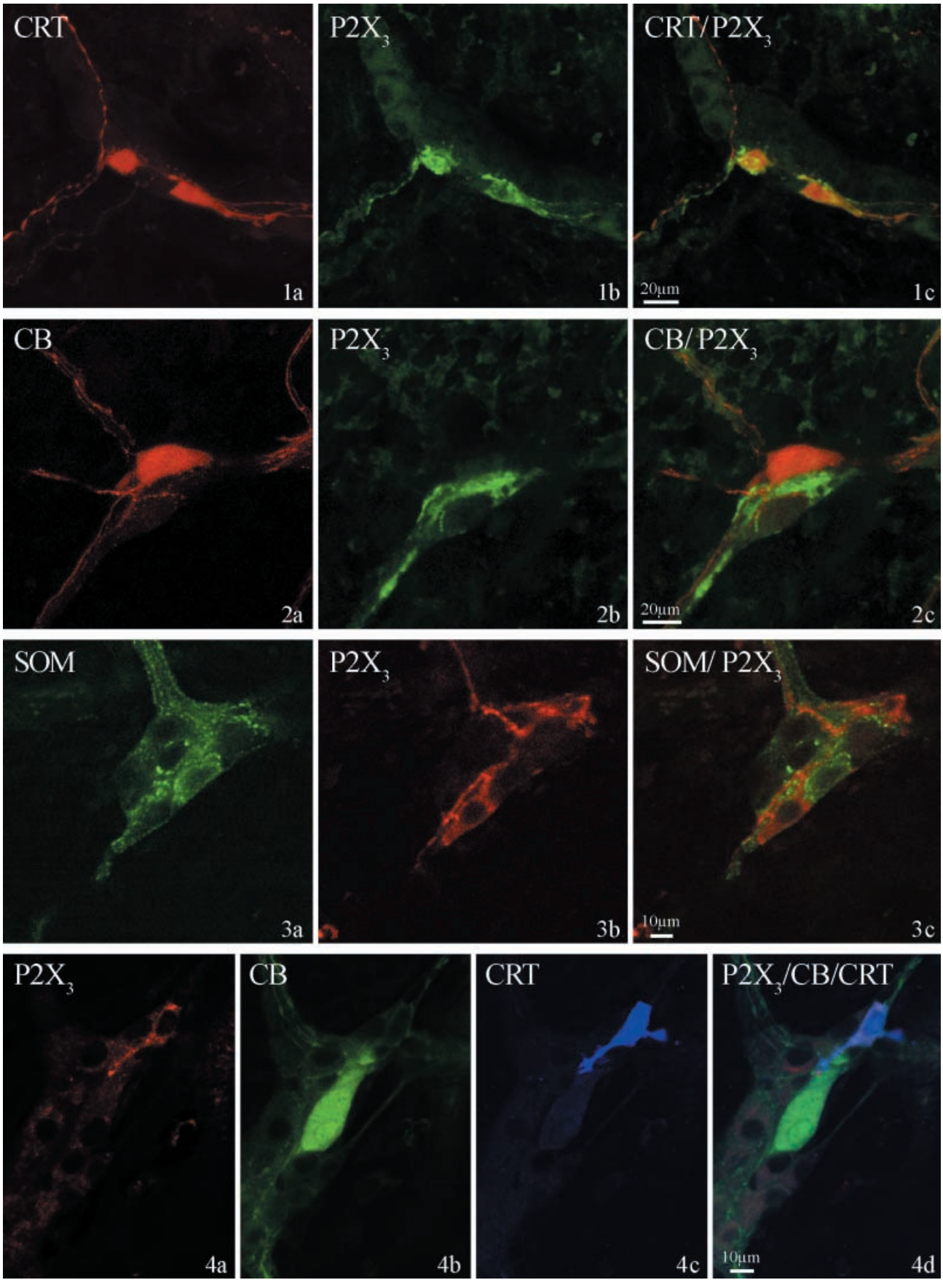
Figs. 1–4 Confocal images of whole-mount preparations of the submucosa ▶

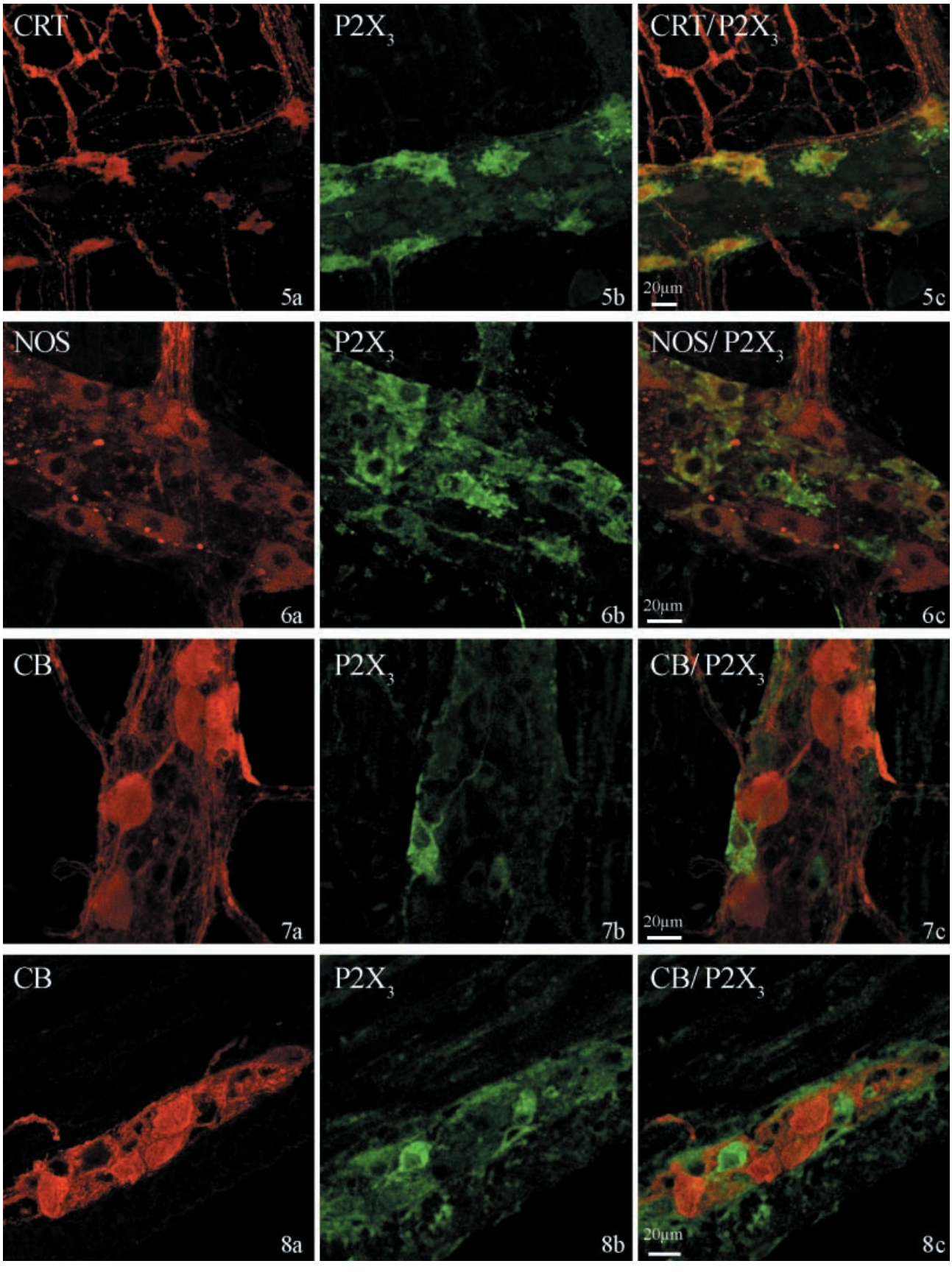
Fig. 1 Double immunolabelling with antibodies directed against the $P2X_3$ subunit (**b**) and calretinin (CRT; **a**) revealed CRT-immunoreactive submucous neurons expressing $P2X_3$ (**c**)

Fig. 2 Double immunolabelling with antibodies directed against the $P2X_3$ subunit (**b**) and calbindin (CB; **a**); no CB-immunoreactive submucous neurons expressed $P2X_3$ (**c**)

Fig. 3 Double immunolabelling with antibodies directed against the $P2X_3$ subunit (**b**) and somatostatin (SOM; **a**); no SOM-immunoreactive submucous neurons expressed $P2X_3$ (**c**)

Fig. 4 Confocal image of a submucosal ganglion triple-stained for $P2X_3$ (**a**), CB (**b**) and CRT (**c**). One submucous neuron is shown to display CRT and $P2X_3$ (*purple*), while the CB-immunoreactive neuron (*green*) is $P2X_3$ immunonegative (**d**)


CRT
1a
P2X3
1b
CRT/P2X3
20µm
1c
CB
2a
P2X3
2b
CB/ P2X3
20µm
2c
SOM
3a
P2X3
3b
SOM/ P2X3
10µm
3c
P2X3
4a
CB
4b
CRT
4c
P2X3/CB/CRT
10µm
4d


CRT
5a
P2X3
5b
CRT/P2X3
20µm
5c
NOS
6a
P2X3
6b
NOS/ P2X3
20µm
6c
CB
7a
P2X3
7b
CB/ P2X3
20µm
7c
CB
8a
P2X3
8b
CB/ P2X3
20µm
8c

the submucous plexuses of the guinea-pig ileum. No $P2X_3$ IR could be detected in subepithelial nerve fibres. Control immunostainings, in which the primary antibodies were omitted, did not yield any immunolabelling. Control stainings of the double- and triple-staining methods showed no linking of secondary antibodies with primary antibodies used in previous steps. The results of the quantitative analysis of the whole mounts are shown in Table 3.

In the submucous plexus, 95±2% of all $P2X_3$-positive neurons were found to display calretinin (CRT) IR, and conversely, 97±3% of all CRT-expressing neurons appeared to bear $P2X_3$ (Table 3; Figs. 1, 4). The $P2X_3$-expressing neurons resembled a morphology associated with the filamentous neuron type. Colocalisation of $P2X_3$ receptors with calbindin (CB; Fig. 2), being a marker for the intrinsic primary sensory neurons in the guinea-pig ileum (Furness et al. 1988, 1998), somatostatin (SOM; Fig. 3) or neuropeptide Y (NPY), was not observed. Moreover, the recently reported presence of CB IR in CRT-expressing neurons (Quinson et al. 2001) could not be confirmed (Fig. 4).

In the myenteric plexus, the $P2X_3$-immunopositive neurons resembled a Dogiel type I morphology. Of all $P2X_3$-immunoreactive neurons, 50±7% demonstrated colocalisation with CRT, while 78±7% of all CRT-expressing neurons showed immunostaining for the $P2X_3$ receptor (Table 3; Figs. 5, 12). Another part of the $P2X_3$-immunoreactive neurons (19±5%) appeared to be nitrergic in nature. Conversely, 23±9% of all nitric oxide synthase (NOS)-immunoreactive neurons expressed the $P2X_3$ receptor (Table 3; Fig. 6). Approximately 30% of the $P2X_3$-immunoreactive neurons did not show any immunostaining for CRT or NOS. Colocalisation of the $P2X_3$ receptor with CB (Figs. 7, 8), neurofilament protein (NFP; Fig. 9), NPY, SOM (Fig. 10) or substance P (SP; Fig. 11) was not detected in myenteric neurons either. No colocalisation of CRT and NOS was observed in myenteric neurons (Fig. 12).

Enteroglial cells, visualised with an antibody directed against S100, and interstitial cells of Cajal, identified by means of c-Kit labelling, did not show immunostaining for the $P2X_3$ receptor.

◀ **Figs. 5–7** Confocal images of whole-mount preparations containing the longitudinal muscle and the myenteric plexus

Fig. 5 Some neurons co-expressed (**c**) $P2X_3$ (**b**) and CRT (**a**)

Fig. 6 Some neurons co-expressed (**c**) $P2X_3$ (**b**) and nitric oxide synthase (NOS; **a**)

Fig. 7 Colocalisation of $P2X_3$ (**b**) with CB (**a**) was not observed (**c**)

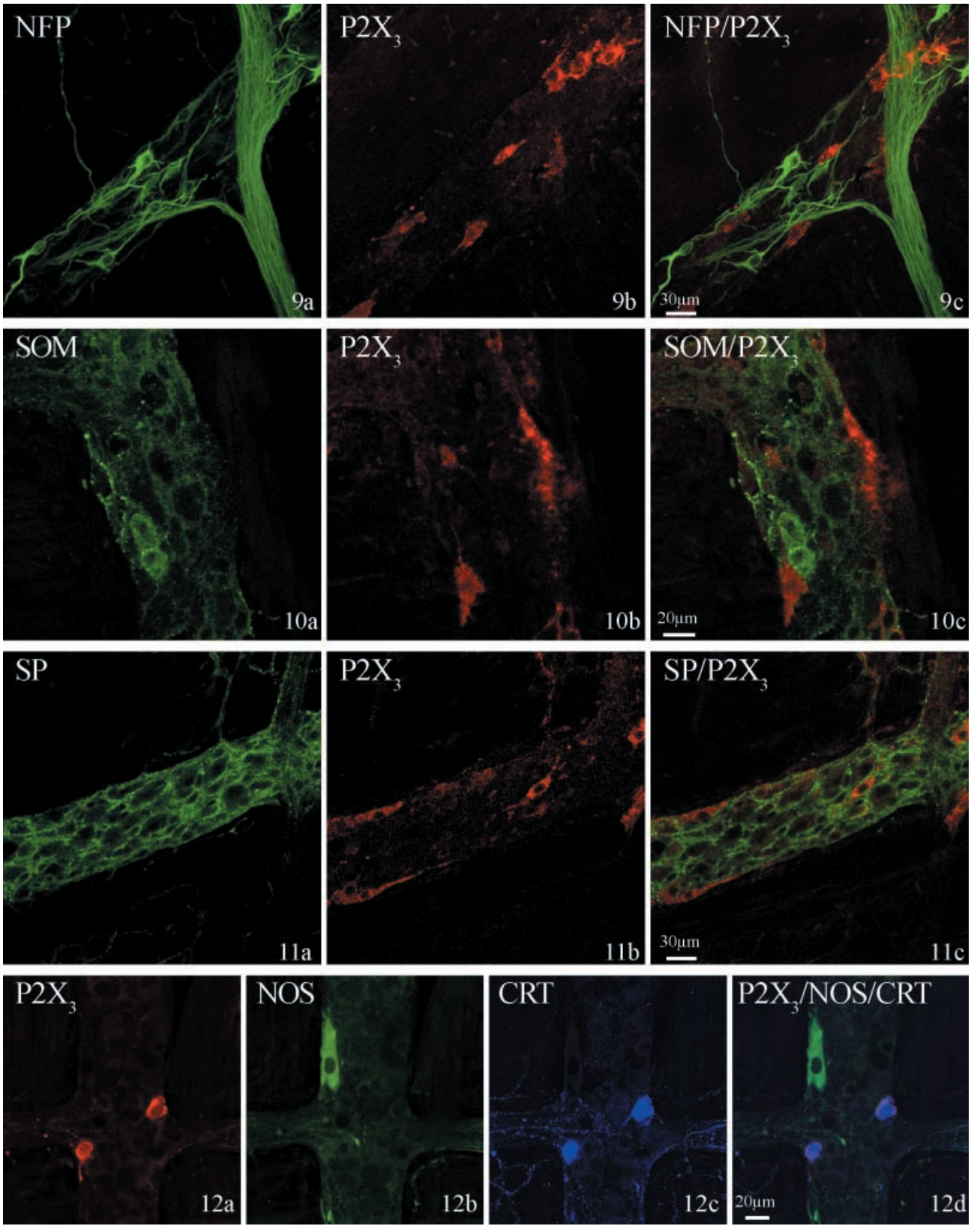
Fig. 8 Cryosection of a myenteric ganglion in the guinea-pig ileum; double immunostaining for CB (**a**) and $P2X_3$ (**b**) did not reveal any colocalisation within myenteric neurons (**c**)

Discussion

To provide the morphological substrate supporting the hypothesis that $P2X_3$ IR is mainly confined to intrinsic sensory neurons within the ENS, we aimed by means of double and triple immunostaining to elucidate the neurochemical coding of $P2X_3$-immunoreactive enteric neurons in the guinea-pig ileum. The latter part of the gastrointestinal (GI) tract was selected for this study because, so far, the most detailed knowledge about the neurochemical coding of the diverse sets of functional enteric neuron classes has been gained from this intestinal region (Costa et al. 1996; Brookes 2001).

In the submucous plexus, $P2X_3$-immunoreactive neurons were found to costain for CRT. Earlier immunocytochemical studies have demonstrated that CRT IR is associated with a population of cholinergic filamentous neurons (Brookes et al. 1991; Brookes 2001). These neurons account for about 12% of all submucous neurons (Song et al. 1992), project to the mucosa and to the submucous arterioles (Brookes et al. 1991), and at least part of them are likely to be among the cholinergic vasodilator neurons (Neild et al. 1990). The finding that almost all CRT-expressing neurons contain $P2X_3$ receptors and, conversely, that virtually all $P2X_3$-immunoreactive neurons show CRT IR, led us to conclude that ca 12% of the submucous neurons express $P2X_3$ receptors. Although a recent study (Quinson et al. 2001) reported a considerable coexistence of CRT and CB in submucous neurons of the guinea-pig ileum, in our study, using the same antibodies, only a faint cytoplasmic and nuclear CB staining could be observed in few of the CRT-immunoreactive neurons provided the CB antiserum was used at a dilution lower than the optimal titer (1:4,000).

In the myenteric plexus, neurons resembling a Dogiel type I morphology express $P2X_3$ receptors. Almost half of all $P2X_3$-expressing neurons are immunoreactive for CRT, while one-fifth are immunoreactive for NOS. No immunostaining for other validated morphofunctional markers such as NFP, SOM, NPY or SP could be observed in the $P2X_3$-immunoreactive neurons. According to the chemical coding of the enteric neuron classes in the guinea-pig small intestine (Costa et al. 1996; Brookes 2001), the neurons exhibiting $P2X_3$/CRT IR can be identified as longitudinal muscle motor neurons, and the neurons expressing $P2X_3$/NOS IR are thought to be short inhibitory circular muscle motor neurons. Longitudinal muscle motor neurons constitute approximately 24% of the neurons in the myenteric plexus of the guinea-pig small intestine (Brookes et al. 1992). They are nearly all immunoreactive for choline acetyltransferase and hence likely to be cholinergic and thus excitatory to the muscle. Approximately 87% of these motor neurons contain CRT. The short-projecting inhibitory circular motor neurons are labelled by VIP, NOS or enkephalin IR, and they make up 14% of the myenteric neurons (Costa et al. 1992, 1996). Given the fact that ca 26% of the myenteric neurons express CRT (Brookes et al. 1991), and that 20–25% are nitrergic (Costa et al. 1992), it can be deduced that approxi-



Figs. 9–12 Confocal images of whole-mount preparations containing the longitudinal muscle and the myenteric plexus

Fig. 9 No myenteric neurons were found to co-express $P2X_3$ (**b**, **c**) and neurofilament protein (NFP; **a**, **c**)

Fig. 10 No myenteric neurons were found to co-express $P2X_3$ (**b**, **c**) and SOM (**a**, **c**)

Fig. 11 No myenteric neurons were found to co-express $P2X_3$ (**b**, **c**) and substance P (SP; **a**, **c**)

Fig. 12 Higher magnification of a myenteric ganglion triple-stained for $P2X_3$ (**a**), NOS (**b**) and CRT (**c**). Myenteric neurons displaying immunoreactivity for $P2X_3$ and CRT (*purple*; **d**)

mately 36±8% of the myenteric neurons express the $P2X_3$ receptor. The neurochemical coding of approximately 30% of the $P2X_3$-immunoreactive myenteric neurons in the guinea-pig ileum remains to be determined. In contrast to what had been put forward in view of the established correlation between the $P2X_3$ receptor and sensory/nociceptive pathways, myenteric CB-immunoreactive Dogiel type II neurons, which are assumed to be the main population of intrinsic sensory neurons within the GI tract, appeared to be immunonegative for the $P2X_3$ receptor. The low number of myenteric neurons responding to an ATP-evoked, rapidly activating and desensitising inward current which was thought to be mediated by $P2X_1$/$P2X_3$ receptors (Zhou and Galligan 1996), are probably not enteric sensory neurons.

In a recent study (Castelucci et al. 2002), strong expression of $P2X_2$ receptors was confined to about 25% of myenteric and 50% of submucosal neurons in the guinea-pig ileum. The $P2X_2$ receptor was shown to be expressed by specific subtypes of enteric neurons. In the submucous plexus, all CB-immunoreactive intrinsic sensory neurons and all vasoactive intestinal polypeptide-immunoreactive non-cholinergic secretomotor neurons were found to express $P2X_2$ IR. In the myenteric plexus, 90% of the CB-expressing intrinsic primary afferent neurons and 91% of the NOS-expressing inhibitory motor neurons costain for $P2X_2$. From these data and our results, it can be deduced that in the myenteric plexus of the guinea-pig ileum, at least 14% of the NOS-expressing neurons, being short inhibitory muscle motor neurons, contain the homomeric $P2X_2$ and $P2X_3$ receptors and/or the heteromeric $P2X_{2/3}$ receptor.

The $P2X_3$ receptor was shown to be expressed predominantly on nociceptive neurons in sensory ganglia (Vulchanova et al. 1996, 1997; Cook et al. 1997; Bradbury et al. 1998; Llewellyn-Smith and Burnstock 1998; Burnstock 2000). This finding has led to the conclusion that ATP contributes to the transduction of painful stimuli and the idea that blockers of $P2X_3$ receptors may have analgesic potency (Burnstock and Wood 1996). However, when determining the expression of $P2X_1$, $P2X_2$ and $P2X_3$ subunits in rat primary afferents, Petruska et al. (2000) reported the predominant presence of the $P2X_1$ subunit in small-diameter sensory neurons, and an extensive overlap of $P2X_1$ IR with neuropeptide expression, particularly SP and calcitonin gene-related peptide, indicating a functional role of $P2X_1$ in nociception. While many of the $P2X_1$ neurons lacked $P2X_3$ IR, Petruska et al. (2000) observed sizable populations of neurons that either coexpressed $P2X_1$ and $P2X_3$, or expressed $P2X_3$ alone. The $P2X_1$ and $P2X_3$ subunits share many characteristics (Dunn et al. 2001; Khakh et al. 2001). These two receptor subunits, assembled as homomers or heteromers with other P2X subunits, may have significant roles in sensory functions, most particularly nociception. These roles are likely to be varied, and each may participate in different forms of nociception (Tsuda et al. 1999). Other researchers suggested that the heteromeric $P2X_{2/3}$ receptor also contributes to nociception (Irnich et al. 2001; Pankratov et al. 2001). Therefore, it seems more likely that in the guinea-pig ileum, not primarily the $P2X_3$ receptor but also other purinergic receptors might be involved in the intrinsic mechanosensory intestinal transduction pathway.

On the other hand, Bian et al. (2000) observed in the guinea-pig ileum that P2X receptors play a significant role in the synaptic transmission from descending interneurons to inhibitory motor neurons of the descending inhibitory reflex pathway, but are not involved in the transmission from distension-sensitive intrinsic sensory neurons to inhibitory motor neurons. In addition, ascending and descending excitatory reflex pathways activated by mechanical stimulation of the mucosa, have also been recently reported in the guinea-pig ileum (Spencer et al. 2000), suggesting that P2 receptors are involved in the neuro-neuronal transmission between interneurons and from interneurons to motor neurons in these pathways. These electrophysiological data might corroborate our present findings favouring the hypothesis that $P2X_3$ receptors are involved in other than viscerosensitive/nociceptive pathways as previously assumed, given their absence on Dogiel type II neurons but their presence on uniaxonal motor neurons to the muscle.

In summary, the present study reveals that the $P2X_3$ receptor is not expressed by intrinsic primary sensory neurons in the guinea-pig ileum. In the submucous plexus, the $P2X_3$ receptor is present in putative vasodilator neurons, while in the myenteric plexus, the receptor is localised on neurons which can be neurochemically identified as longitudinal muscle motor neurons and short inhibitory circular muscle motor neurons. The present data do not point to an involvement of an intrinsic $P2X_3$-mediated mechanosensory transduction pathway in the guinea-pig small intestine. However, this does not negate the possibility that extrinsic sensory nerves expressing $P2X_3$ receptors are involved in a purinergic mechanosensory transduction pathway which was proposed for several tube- and sac-like visceral organs (Burnstock 1999). Evidence for such an involvement is already available for the urinary bladder of rabbits (Ferguson et al. 1997), rats (Jiang and Morrison 1996; Namasivayam et al. 1999) and mice (Cockayne et al. 2000; Vlaskovska et al. 2001), and for the ureter of the guinea-pig (Knight et al. 2002).

Acknowledgements This study was supported by the IAP grants P4/16 and P5/20 of the Federal Belgian Government.

References

Barajas-López C, Huizinga JD, Collins SM, Gerzanich V, Espinosa-Luna R, Peres AL (1996) P_{2X}-purinoceptors of myenteric neurones from the guinea-pig ileum and their unusual pharmacological properties. Br J Pharmacol 119:1541–1548

Berthoud H-R, Neuhuber WL (2000) Functional and chemical anatomy of the afferent vagal system. Auton Neurosci 85:1–17

Bian X, Bertrand PP, Bornstein JC (2000) Descending inhibitory reflexes involve P2X receptor-mediated transmission from interneurons to motor neurons in guinea-pig ileum. J Physiol 528:551–560

Bradbury EJ, Burnstock G, McMahon SB (1998) The expression of $P2X_3$ purinoceptors in sensory neurons: effects of axotomy and glial-derived neurotrophic factor. Mol Cell Neurosci 12: 256–268

Brookes SJH (2001) Classes of enteric nerve cells in the guinea-pig small intestine. Anat Rec 262:58–70

Brookes SJH, Steele PA, Costa M (1991) Calretinin immunoreactivity in cholinergic motor neurones, interneurones and vasomotor neurones in the guinea-pig small intestine. Cell Tissue Res 263:471–481

Brookes SJH, Song ZM, Steele PA, Costa M (1992) Identification of motor neurons to the longitudinal muscle of the guinea pig ileum. Gastroenterology 103:961–973

Brouns I, Adriaensen D, Burnstock G, Timmermans J-P (2000) Intraepithelial vagal sensory nerve terminals in rat pulmonary neuroepithelial bodies express $P2X_3$ receptors. Am J Respir Cell Mol Biol 23:52–61

Brouns I, Van Nassauw L, Van Genechten J, Majewski M, Scheuermann DW, Timmermans J-P, Adriaensen D (2002) Triple immunofluorescence staining with antibodies raised in the same species to study the complex innervation pattern of intrapulmonary chemoreceptors. J Histochem Cytochem 50:575–582

Burnstock G (1999) Release of vasoactive substances from endothelial cells by shear stress and purinergic mechanosensory transduction. J Anat 194:335–342

Burnstock G (2000) P2X receptors in sensory neurones. Br J Anaesth 84:476–488

Burnstock G (2001a) Expanding field of purinergic signaling. Drug Dev Res 52:1–10

Burnstock G (2001b) Purinergic signalling in gut. In: Abbracchio MP, Williams M (eds) Handbook of experimental pharmacology, vol 152/II. Purinergic and pyrimidinergic signalling. II. Cardiovascular, respiratory, immune, metabolic and gastrointestinal tract function. Springer, Berlin Heidelberg New York, pp 141–238

Burnstock G (2001c) Purine-mediated signalling in pain and visceral perception. Trends Pharmacol Sci 22:182–188

Burnstock G, Wood JN (1996) Purinergic receptors: their role in nociception and primary afferent neurotransmission. Curr Opin Neurobiol 6:526–532

Castelucci P, Robbins HL, Poole DP, Furness JB (2002) The distribution of purine $P2X_2$ receptors in the guinea-pig enteric nervous system. Histochem Cell Biol 117:415–422

Cervero F (1994) Sensory innervation of the viscera: peripheral basis of visceral pain. Physiol Rev 74:95–138

Cockayne DA, Hamilton SG, Zhu Q-M, Dunn PM, Zhong Y, Novakovic S, Malmberg AB, Cain G, Berson A, Kassotakis L, Hedley L, Lachnit WG, Burnstock G, McMahon SB, Ford APDW (2000) Urinary bladder hyporeflexia and reduced pain-related behaviour in $P2X_3$-deficient mice. Nature 407:1011–1015

Cook SP, Vulchanova L, Hargreaves KM, Elde R, McCleskey EW (1997) Distinct ATP receptors on pain-sensing and stretch-sensing neurons. Nature 387:505–508

Costa M, Furness JB, Pompolo S, Brookes SJH, Bornstein JC, Bredt DS, Snyder SH (1992) Projections and chemical coding of neurons with immunoreactivity for nitric oxide synthase in the guinea-pig small intestine. Neurosci Lett 148:121–125

Costa M, Brookes SJH, Steele PA, Gibbins I, Burcher E, Kandiah CJ (1996) Neurochemical classification of myenteric neurons in the guinea-pig ileum. Neuroscience 75:949–967

Dunn PM, Zhong Y, Burnstock G (2001) P2X receptors in peripheral neurons. Prog Neurobiol 65:107–134

Facer P, Knowles CH, Tam PKH, Ford AP, Dyer N, Baecker PA, Anand P (2001) Novel capsaicin (VR1) and purinergic ($P2X_3$) receptors in Hirschsprung's intestine. J Pediatr Surg 36:1679–1684

Ferguson DR, Kennedy I, Burton TJ (1997) ATP is released from rabbit epithelial cells by hydrostatic pressure changes: a possible sensory mechanism? J Physiol 505:503–511

Furness JB, Keast JR, Pompolo S, Bornstein JC, Costa M, Emson PC, Lawson DEM (1988) Immunohistochemical evidence for the presence of calcium-binding proteins in enteric neurons. Cell Tissue Res 252:79–87

Furness JB, Pompolo S, Murphy R, Giraud A (1989) Projections of neurons with neuromedin U-like immunoreactivity in the small intestine of the guinea-pig. Cell Tissue Res 257:415–422

Furness JB, Kunze WAA, Bertrand PP, Clerc N, Bornstein JC (1998) Intrinsic primary afferent neurons of the intestine. Prog Neurobiol 54:1–18

Gröschel-Stewart U, Bardini M, Robson T, Burnstock G (1999) P2X receptors in the rat duodenal villus. Cell Tissue Res 296:599–605

Grundy D, Scratcherd T (1989) Sensory afferents from the gastrointestinal tract. In: Schultz SG(ed) Handbook of physiology, section 6. The gastrointestinal system, vol 1. Motility and circulation, part 1. Am Physiol Soc, Bethesda, pp 593–620

Harboe N, Ingild A (1973) Immunization, isolation of immunoglobulins, estimation of antibody titre. Scand J Immunol (Suppl) 1:161–164

Hens J, Schrödl F, Brehmer A, Adriaensen, Neuhuber W, Scheuermann DW, Schemann M, Timmermans J-P (2000) Mucosal projections of enteric neurons in the porcine small intestine. J Comp Neurol 421:429–436

Hens J, Vanderwinden JM, Delaet MH, Scheuermann DW, Timmermans J-P (2001) Morphological and neurochemical identification of enteric neurones with mucosal projections in the human small intestine. J Neurochem 76:464–471

Hu H-Z, Gao N, Lin Z, Gao C, Liu S, Ren J, Xia Y, Wood JD (2001) $P2X_7$ receptors in the enteric nervous system of guinea-pig small intestine. J Comp Neurol 440:299–310

Hubscher CH, Petruska JC, Rau KK, Johnson RD (2001) Co-expression of P2X receptor subunits on rat nodose neurons that bind the isolectin GS-I-B_4. Neuroreport 12:2995–2997

Irnich D, Burgstahler R, Grafe P (2001) P2 nucleotide receptors in peripheral nerve trunk. Drug Dev Res 52:83–88

Jänig W, Koltzenburg M (1988) The neural basis of consciously perceived sensations from the gut. In: Sieger MV, Goebell H (eds) Nerves and the gastrointestinal tract. Kluwer Academic, Dortrecht, pp 383–398

Jiang W, Morrison JFB (1996) Sensitization of pelvic nerve afferent neurones from the rat bladder. J Auton Nerv Syst 58:187–188

Khakh BS, Burnstock G, Kennedy C, King BF, North RA, Séguéla P, Voigt M, Humphrey PPA (2001) International union of pharmacology. XXIV. Current status of the nomenclature and properties of P2X receptors and their subunits. Pharmacol Rev 53:107–118

Kirchgessner AL, Tamir H, Gershon MD (1992) Identification and stimulation by serotonin of intrinsic sensory neurons of the submucosal plexus of the guinea pig gut: activity-induced expression of Fos immunoreactivity. J Neurosci 12:235–248

Knight GE, Bodin P, De Groat WC, Burnstock G (2002) ATP is released from guinea pig ureter epithelium on distension. Am J Physiol Renal Physiol 282:F281–F288

LePard KJ, Messori E, Galligan JJ (1997) Purinergic fast excitatory postsynaptic potentials in myenteric neurons of guinea pig: distribution and pharmacology. Gastroenterology 113:1522–1534

Llewellyn-Smith IJ, Burnstock G (1998) Ultrastructural localization of $P2X_3$ receptors in rat sensory neurons. Neuroreport 9:2245–2250

Llewellyn-Smith IJ, Costa M, Furness JB (1985) Light and electron microscopic immunocytochemistry of the same nerves from whole mount preparations. J Histochem Cytochem 33:857–866

Meedeniya AB, Brookes SJH, Hennig GW, Costa M (1998) The projections of 5-hydroxytryptamine-accumulating neurones in the myenteric plexus of the small intestine of the guinea-pig. Cell Tissue Res 291:375–384

Namasivayam S, Eardley I, Morrison JFB (1999) Purinergic sensory neurotransmission in the urinary bladder: an in vitro study in the rat. BJU Int 84:854–860

Neild TO, Shen KZ, Surprenant A (1990) Vasodilatation of arterioles by acetylcholine released from single neurones in the guinea-pig submucosal plexus. J Physiol 420:247–265

Neunlist M, Schemann M (1997) Projections and neurochemical coding of myenteric neurons innervating the mucosa of the guinea pig proximal colon. Cell Tissue Res 287:119–125

Neunlist M, Dobreva G, Schemann M (1999) Characteristics of mucosally projecting myenteric neurones in the guinea-pig proximal colon. J Physiol 517:533–546

Oglesby IB, Lachnit WG, Burnstock G, Ford APDW (1999) Subunit specificity of polyclonal antisera to the carboxy terminal regions of P2X receptors, $P2X_1$ through $P2X_7$. Drug Dev Res 47:189–195

Pankratov YV, Lalo UV, Dashkin AN, Krishtal OA (2001) Heterogeneity of the functional expression of $P2X_3$ and $P2X_{2/3}$ receptors in primary nociceptive neurons of rat. Neurochem Res 26:993–1000

Petruska JC, Cooper BY, Gu JG, Rau KK, Johnson RD (2000) Distribution of $P2X_1$, $P2X_2$, and $P2X_3$ receptor subunits in rat primary afferents: relation to population markers and specific cell types. J Chem Neuroanat 20:141–162

Quinson N, Robbins HL, Clark MJ, Furness JB (2001) Calbindin immunoreactivity of enteric neurons in the guinea-pig ileum. Cell Tissue Res 305:3–9

Shindler KS, Roth KA (1996) Double immunofluorescent staining using two unconjugated primary antisera raised in the same species. J Histochem Cytochem 44:1331–1335

Song ZM, Brookes SJH, Steele PA, Costa M (1991) Identification of myenteric neurons which project to the mucosa of the guinea-pig small intestine. Neurosci Lett 129:294–298

Song ZM, Brookes SJH, Steele PA, Costa M (1992) Projections and pathways of submucous neurons to the mucosa of the guinea-pig small intestine. Cell Tissue Res 269:87–98

Song ZM, Brookes SJH, Costa M (1994) All calbindin-immunoreactive myenteric neurons project to the mucosa of the guinea-pig small intestine. Neurosci Lett 180:219–222

Spencer NJ, Walsh M, Smith TK (2000) Purinergic and cholinergic neuro-neuronal transmission underlying reflexes activated by mucosal stimulation in the isolated guinea-pig ileum. J Physiol 522:321–331

Tsuda M, Ueno S, Inoue K (1999) In vivo pathway of thermal hyperalgesia by intrathecal administration of alpha,beta-methylene ATP in mouse spinal cord: involvement of the glutamate-NMDA receptor system. Br J Pharmacol 127:449–456

Van Nassauw L, Bogers J, Van Marck E, Timmermans J-P (2001) Role of reactive nitrogen species in neuronal cell damage during intestinal schistosomiasis. Cell Tissue Res 303:329–336

Vlaskovska M, Kasakov L, Rong W, Bodin P, Bardini M, Cockayne DA, Ford APDW, Burnstock G (2001) Differential distribution of two ATP-gated channels (P2X receptors) determined by immunocytochemistry. Proc Natl Acad Sci U S A 93:8063–8067

Vulchanova L, Arvidsson U, Riedl M, Wang J, Buell G, Surprenant A, North RA, Elde R (1996) Differential distribution of two ATP-gated ion channels (P_{2X} receptors) determined by immunocytochemistry. Proc Natl Acad Sci U S A 93:8063–8067

Vulchanova L, Riedl M, Shuster SJ, Buell G, Surprenant A, North RA, Elde R (1997) Immunocytochemical study of the $P2X_2$ and $P2X_3$ receptor subunits in rat and monkey sensory neurons and their central terminals. Neuropharmacology 36:1229–1242

Xiang Z, Bo X, Burnstock G (1998a) Localization of ATP-gated P2X receptor immunoreactivity in rat sensory and sympathetic ganglia. Neurosci Lett 256:105–108

Xiang Z, Bo X, Oglesby IB, Ford APDW, Burnstock G (1998b) Localization of ATP-gated $P2X_2$ receptor immunoreactivity in the rat hypothalamus. Brain Res 813:390–397

Yiangou Y, Facer P, Birch R, Sangameswaran L, Eglen R, Anand P (2000) $P2X_3$ receptor in injured human sensory neurons. Neuroreport 11:993–996

Yiangou Y, Facer P, Baecker PA, Ford AP, Knowles CH, Chan CLH, Williams NS, Anand P (2001) ATP-gated ion channel $P2X_3$ is increased in human inflammatory bowel disease. Neurogastroenterol Motil 13:365–369

Zhou X, Galligan JJ (1996) P2X purinoceptors in cultured myenteric neurons of guinea-pig small intestine. J Physiol 496:719–729