Cell Tissue Res (2005) 319: 191–200
DOI 10.1007/s00441-004-1002-7

REGULAR ARTICLE

Huai Zhen Ruan · Geoffrey Burnstock

The distribution of $P2X_5$ purinergic receptors in the enteric nervous system of mouse

Received: 8 December 2003 / Accepted: 10 September 2004 / Published online: 18 November 2004

Abstract The distribution of the $P2X_5$ purinoceptor in the enteric nervous system of the mouse was studied by immunohistochemistry. $P2X_5$ receptor immunoreactivity was widely distributed in myenteric and submucosal plexuses throughout the gastrointestinal tract. In myenteric plexuses, immunoreactivity for the $P2X_5$ receptor was observed in nerve fibres that enveloped ganglion cell bodies, and possibly on glial cell processes. $P2X_5$ receptor immunoreactivity was colocalised with vasoactive intestinal peptide and surrounded ganglion cells that contained calretinin, calbindin or nitric oxide synthase. In the submucous plexus, $P2X_5$ receptor immunoreactivity occurred throughout the cytoplasm and on the surface membranes of the nerve cells. Double-labelling studies showed that 22%, 9%, 6% and 68% of $P2X_5$ receptor-immunoreactive neurones were also immunoreactive for calretinin, calbindin, nitric oxide synthase and vasoactive intestinal peptide, respectively. Thus, the $P2X_5$ receptor subunit is expressed in specific functional groups of neurones. $P2X_2$ and $P2X_3$ receptors were also present in the mouse enteric plexuses but no immunoreactivity for $P2X_1$, $P2X_4$ or $P2X_6$ receptors was found.

Keywords Purinergic receptors · ATP · Enteric nervous system · Neurotransmitter · Mouse

H. Z. Ruan
Department of Neurobiology, Third Military Medical University,
Chongqing, 400038, China

H. Z. Ruan · G. Burnstock (✉)
Autonomic Neuroscience Institute, Royal Free & University College Medical School,
Rowland Hill Street,
London, NW3 2PF, UK
e-mail: g.burnstock@ucl.ac.uk
Tel.: +44-207-8302948
Fax: +44-207-8302949

Introduction

ATP evokes responses in neuronal and non-neuronal cells by activating various P2 receptor subtypes (Ralevic and Burnstock 1998). ATP and related purines have been recognised as neurotransmitters in the central, peripheral, and enteric nervous systems (Burnstock 2003). Two families of purinoceptors have been identified: a P2X ionotropic ligand-gated ion channel family and a P2Y metabotropic G-protein-coupled family (Abbracchio and Burnstock 1994). To date, seven mammalian P2X receptor subunits ($P2X_1$–$P2X_7$) have been identified by molecular cloning; these can assemble to form homomeric and heteromeric receptors with different pharmacological properties, including different responses to agonists and antagonists and differences in desensitisation properties (Khakh et al. 2001).

The $P2X_5$ receptor was first cloned from rat coeliac ganglia (Collo et al. 1996). It has been cloned and expressed in heart, brain, spinal cord, peripheral ganglia, retina, endocrine and epithelial cells, adrenal gland, skin, gut and bladder (Collo et al. 1996; Brandle et al. 1998; Gröschel-Stewart et al. 1999a, 1999b; Lee et al. 2000; Dunn et al. 2001; Glass and Burnstock 2001; Greig et al. 2003). Recently, the receptor has been implicated in the differentiation of satellite cells into mature multinucleated muscle fibres (Ryten et al. 2001, 2002). Less is known about the neurobiology of $P2X_5$ receptors in the enteric nervous system (ENS). The homomeric $P2X_5$ receptor is recognised as a slowly desensitising receptor sensitive to ATP and 2-methylthio ATP but insensitive to α,β-methylene ATP.

ATP has been shown to elicit responses of neurones in both plexuses of the ENS, indicating the presence of P2X receptors (for reviews, see Burnstock 2001; Dunn et al. 2001). Application of ATP to enteric neurones evokes a fast-activating depolarisation that is reminiscent of the depolarising action of acetylcholine at nicotinic receptors (Galligan and Bertrand 1994; Barajas-López et al. 1996; Zhou and Galligan 1996). Furthermore, comparisons of agonist potencies and susceptibility to antagonists confirm

that effects on both myenteric and submucosal neurones are mediated through P2X receptors (Barajas-López et al. 1996, 2000). This finding has led to the hypothesis that P2X receptors are one of the mediators of fast excitatory post-synaptic potentials (EPSPs) in the ENS (Galligan and Bertrand 1994; LePard and Galligan 1999; Galligan et al. 2000). However, at present, few selective agonists and antagonists discriminate clearly between subtypes of P2X receptors family. In both myenteric and submucosal neurones, the electrophysiology and pharmacology is ambiguous as to which type of the seven known subtypes of P2X receptor (Ralevic and Burnstock 1998) is activated (Glushakov et al. 1998; Barajas-López et al. 2000).

The presence of P2X immunoreactivity in the ENS has recently been reported but most data has been concerned with the presence of immunoreactivity to $P2X_2$ and $P2X_3$ receptors in guinea-pig, mouse and human ENS (Vulchanova et al. 1996; Facer et al. 2001; Hu et al. 2001; Yiangou et al. 2001; Castelucci et al. 2002; Giaroni et al. 2002; Poole et al. 2002; Van Nassauw et al. 2002; Bian et al. 2003; De Man et al. 2003; Ren et al. 2003). The $P2X_2$ receptor is expressed by specific subtypes of enteric neurones, including inhibitory motor neurones, non-cholinergic secretomotor neurones and intrinsic primary afferent neurones (Castelucci et al. 2002). The $P2X_3$ receptor subunit is expressed in excitatory and inhibitory motor neurones to muscle, ascending interneurones and cholinergic secretomotor neurones (Poole et al. 2002; Van Nassauw et al. 2002; Bian et al. 2003). $P2X_7$ immunoreactivity has been found in nerve fibres and somata in both the myenteric plexuses (MPs) and the submucous plexuses (SMPs) of the guinea-pig small intestine (Hu et al. 2001). However, no studies of the involvement of the $P2X_5$ receptor subtype have been reported for the ENS, apart from one report showing $P2X_5$ receptor localisation on interstitial cells of Cajal (Burnstock and Lavin 2002). In the present work, we have used an antiserum specific to the $P2X_5$ receptor subtype in order to study the distribution of this receptor in the ENS of the mouse.

Materials and methods

Animals

The 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 and in accordance with the Animals (Scientific Procedures) Act, 1986, governing the use of animals. All protocols were approved by the local animal ethics committee. A total of 16 T.O adult mice of both sexes, 19–25 g in weight, were used in this study. They were killed by asphyxiation with a rising concentration of CO_2 (between 0% and 100%) and death was confirmed by cervical dislocation. Thereafter, animals were perfused immediately through the heart (left ventricle) with 50 ml 0.9% NaCl solution followed by fixative containing 4% paraformaldehyde, 0.2% saturated picric acid in 0.1 M phosphate-buffered saline (PBS, pH 7.4).

Whole-mount preparations

Segments of stomach, jejunum, ileum and colon were quickly removed and washed with PBS. An incision was made along the midline of the gut and the tissue was pinned as a flat sheet onto Sylgard (Dow Corning, Wiesbaden, Germany) with the mucosa face-down. Tissue samples were immersed in the same fixative (4% paraformaldehyde, 0.2% saturated picric acid in 0.1 M PBS) overnight at 4 °C, unpinned, and washed in PBS (3×10 min). After being cleared, 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 MP attached. In a few whole-mounts, the circular muscle was only partially removed. In the whole-mounts containing the SMP, the mucosa was scraped off by using a blunt scalpel. The whole-mounts were stored in PBS containing 0.1% sodium azide.

Primary antibody directed against $P2X_5$ receptor

The antibody directed against the $P2X_5$ receptor, raised by Roche Bioscience (Palo Alto, Calif., USA), was as detailed previously (Oglesby et al. 1999). The specificity of the $P2X_5$ 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 was 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 immunoreactivity (Xiang et al. 1998).

Immunocytochemistry

The antisera used in this study and their respective dilutions are listed in Table 1. The preparations were washed 3×5 min in PBS and then preincubated in 10% normal horse serum (NHS), 0.2% Triton X-100 in PBS for 30 min. Preparations were subsequently incubated in the primary antibodies, diluted with 10% NHS in PBS containing 0.05% merthiolate and 0.2% Triton X-100, overnight and then in Cy3-conjugated donkey anti-rabbit IgG, diluted 1:300 in 1% NHS in PBS containing 0.05% merthiolate, for 1 h. All incubations were carried out at room temperature and separated by 3×5 min washes in PBS. The preparations were mounted with Citifluor (Citifluor, London, UK) and examined by fluorescence microscopy.

To demonstrate the colocalisation of the $P2X_5$ receptor with calretinin, calbindin, neuronal nitric oxide synthase

Table 1 Antisera used for immunocytochemistry

Antigen	Host	Dilution	Source
Primary antisera			
$P2X_5$ receptor	Rabbit	1:400	Roche Bioscience, Palo Alto, Calif., USA
$P2X_1$ receptor	Rabbit	1:200	Roche Bioscience
$P2X_2$ receptor	Rabbit	1:400	Roche Bioscience
$P2X_3$ receptor	Rabbit	1:400	Roche Bioscience
$P2X_4$ receptor	Rabbit	1:200	Roche Bioscience
$P2X_6$ receptor	Rabbit	1:200	Roche Bioscience
Calretinin	Mouse	1:2,000	Swant, Bellinzola, Switzerland
Calbindin D-28	Mouse	1:2,000	Swant
Neuronal nitric oxide synthase	Sheep	1:800	Santa Cruz Biotechnology, Santa Cruz, Calif., USA
Vasoactive intestinal peptide	Mouse	1:400	Biogenesis, Sandown, USA
Secondary antisera			
Cy3-conjugated donkey anti-rabbit IgG		1:300	Jackson ImmunoResearch Lab, West Grove, Pa., USA
Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG		1:200	Jackson ImmunoResearch Lab
FITC-conjugated donkey anti-sheep IgG		1:200	Jackson ImmunoResearch Lab

(NOS) and vasoactive intestinal peptide (VIP), preparations were immunostained for the $P2X_5$ receptor, as above, and then incubated with these antibodies overnight. Subsequently, the preparations were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG or FITC-conjugated donkey anti-sheep IgG. All the incubations and reactions were carried out at room temperature and separated by 3×10 min washes in PBS. The preparations were mounted with Citifluor and examined by fluorescence microscopy.

The following control experiments were performed to establish the specificity of the immunoreactivity: omission of the primary antibodies and preabsorptions of the primary antibodies with their homologous peptide antigens.

Photomicroscopy

Images of immunofluorescence labelling were taken with a Leica DC 200 digital camera (Leica, Switzerland) attached to a Zeiss Axioplan microscope (Zeiss, Germany). Filter sets included the following: for Cy3, 510–550 nm excitation, 590 nm emission; 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.

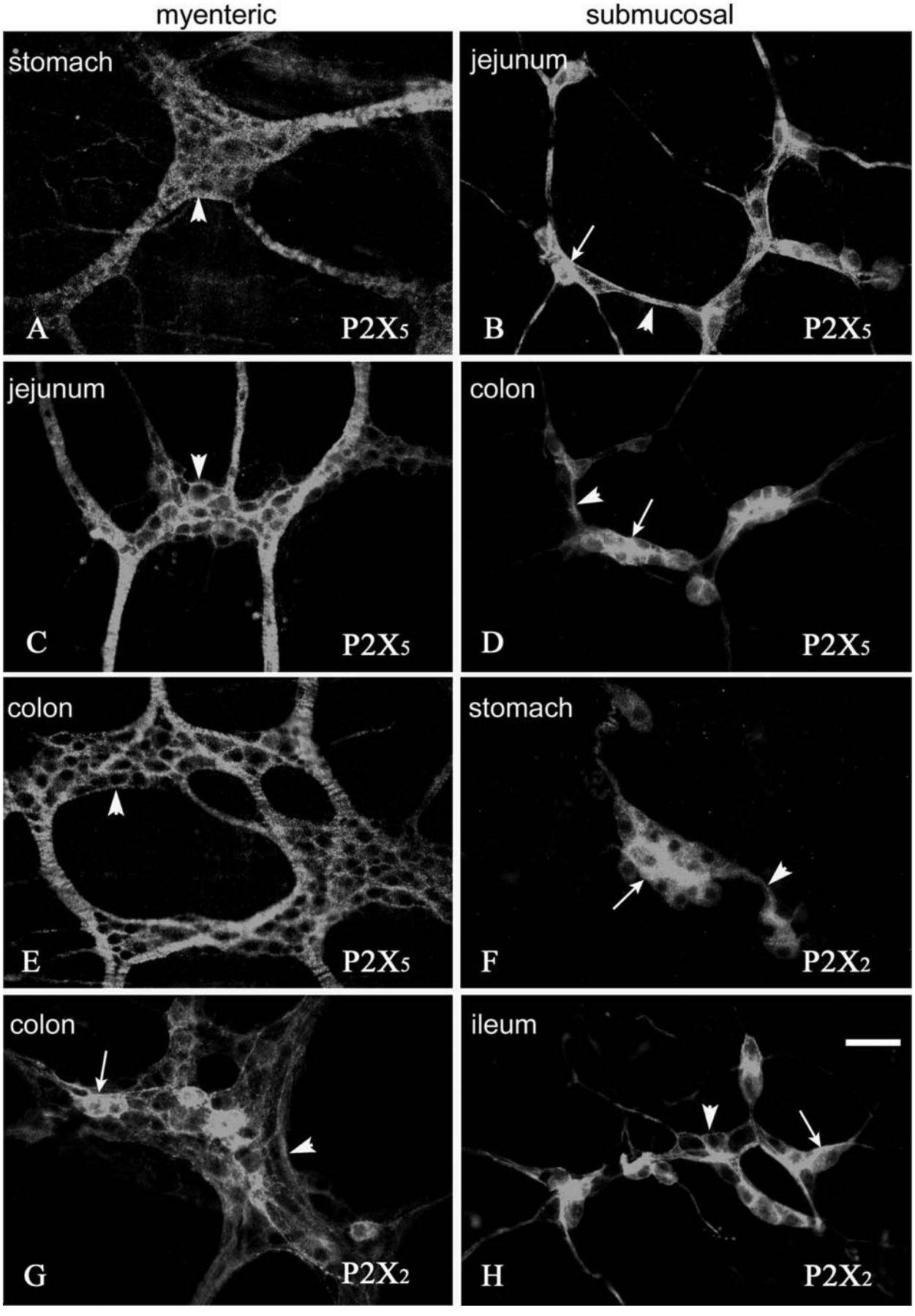
Analysis

All analyses were performed at ×20 objective magnification. The whole-mount preparations were also used to perform a quantitative analysis as described previously (Van Nassauw et al. 2001). Briefly, the immunoreactive-positive neurone bodies in the submucosal and myenteric ganglia were counted per visual field (0.3 mm^2) in the whole-mount preparations. Ten randomly chosen fields in each whole-mount preparation were analysed and the number of immunoreactive neurones was calculated.

Results

$P2X_5$ receptor immunoreactivity in MPs and SMPs

$P2X_5$ receptor immunoreactivity was present in all regions of the mouse gastrointestinal tract examined: the MPs and SMPs of the stomach, jejunum, ileum and colon. The distribution of $P2X_5$ receptor immunoreactivity was similar in the various regions of the gastrointestinal tract. In the MP, immunoreactivity was predominantly localised in nerve fibres running through the ganglia and enveloping neuronal cell bodies (Figs. 1, 2), although staining on glial cell processes could not be excluded. No $P2X_1$, $P2X_4$ or $P2X_6$ receptor immunoreactivity was found in the MP. Weaker $P2X_3$ receptor immunoreactivity was found in the MP. On the other hand, $P2X_2$ receptor immunoreactivity was abundant in myenteric ganglia (Figs. 1, 2). High levels of $P2X_5$ immunoreactivity were found in the SMP in neurone cell bodies, nerve fibres and nerve bundles. Immunoreactivity occurred throughout the cytoplasm and on the surface membranes of the nerve cells. The strongest $P2X_5$ immunoreactivity was found to be associated with ganglion cell bodies, especially on the neuronal membranes (Figs. 1, 2). $P2X_5$-immunoreactive nerve fibres surrounded the ganglion cell bodies of essentially all of the SMP neurones. Immunoreactivity for $P2X_2$ and $P2X_3$ receptors, but not for $P2X_1$, $P2X_4$ or $P2X_6$ receptors, was present in submucosal ganglia (Fig. 1; $P2X_3$ receptor data not shown). Control immunostaining, in which the


myenteric
submucosal
stomach
A
P2X5
jejunum
B
P2X5
jejunum
C
P2X5
colon
D
P2X5
colon
E
P2X5
stomach
F
P2X2
colon
G
P2X2
ileum
H
P2X2

◀ **Fig. 1** Examples of $P2X_5$ and $P2X_2$ receptor immunoreactivity in various regions of the mouse gastrointestinal tract. $P2X_5$ receptor immunoreactivity in the myenteric plexus (MP) of stomach (**A**), in MPs and submucosal plexuses (SMP) of the jejunum (**B**, **C**), in MPs and SMPs of the colon (**D**, **E**), in the SMP of the stomach (**F**), in the MP of the colon (**G**) and in the SMP of the ileum (**H**). Note the positive staining for $P2X_2$ receptors, in both cell bodies (*arrows*) and nerve fibres (*arrowheads*). *Bar* 50 μm

primary antibodies were omitted or pre-absorbed with the relevant peptide, did not yield immunolabelling.

Double-labelling studies

Double-labelling studies were conducted to identify major classes of neurones that had strong $P2X_5$ receptor immunoreactivity in the myenteric and submucosal ganglia of the ileum (Figs. 3, 4). In the MP of ileum, double-labelling for the $P2X_5$ receptor and calretinin, calbindin or NOS revealed that $P2X_5$ receptor immunoreactivity was colocalised with and surrounded neurones that expressed these neurochemical markers (Fig. 3). Many of the nerve fibres in the MP of the ileum were immunoreactive for $P2X_5$ and VIP (Fig. 3).

Both the number and percentage of neurones colocalising with various markers are summarised in Table 2. In the SMP of ileum, about 1/5 of all $P2X_5$-positive neurones were found to display calretinin immunoreactivity but, conversely, most of the calretinin-expressing neurones appeared to bear $P2X_5$ receptor immunoreactivity (Table 2; Fig. 3). Colocalisation of $P2X_5$ receptors with calbindin (Table 2; Fig. 3) was observed. Of the $P2X_5$-immunoreactive cells, a few were also calbindin-immunoreactive. Some NOS-immunoreactive cells were present in the SMP; however, almost all NOS-immunoreactive cells were also positive for the $P2X_5$ subunit. Conversely, few $P2X_5$ -positive neurones expressed NOS (Table 2; Fig. 3). Double-immunofluorescent histochemistry showed that about 90% of VIP-immunoreactive nerve cells were $P2X_5$ receptor-immunoreactive in the submucosal ganglia. On the other hand, about 70% $P2X_5$-immunoreactive submucosal nerve cells were immunoreactive for VIP (Table 2; Fig. 3).

Discussion

P2X receptors contribute to fast synaptic excitation in the ENS (Galligan and Bertrand 1994; Johnson et al. 1999). High concentrations of ATP in subpopulations of myenteric neurones in various regions of the gut of the guinea-pig, rabbit and rat have been reported (Crowe and Burnstock 1981; Belai and Burnstock 1994). In addition, after application of an appropriate stimulus, ATP is released from enteric nerves (Burnstock et al. 1978; McConalogue et al. 1996) and the subsequent activation of specific receptors on enteric neurones and muscle cells may evoke either the excitation or the inhibition of smooth muscle function (Burnstock 2001). Furthermore, a comparison of agonist potencies and susceptibility to antagonists supports the contention that synaptic transmission in both myenteric and submucosal neurones is mediated

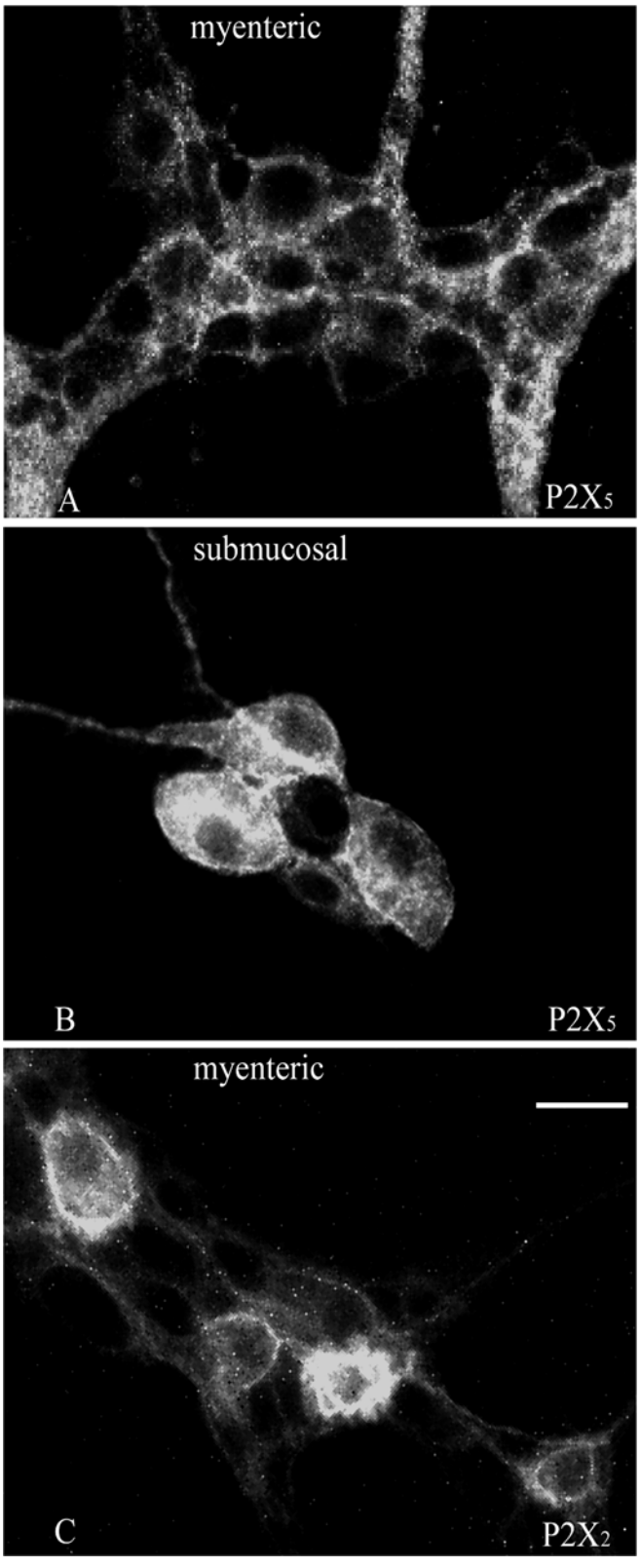
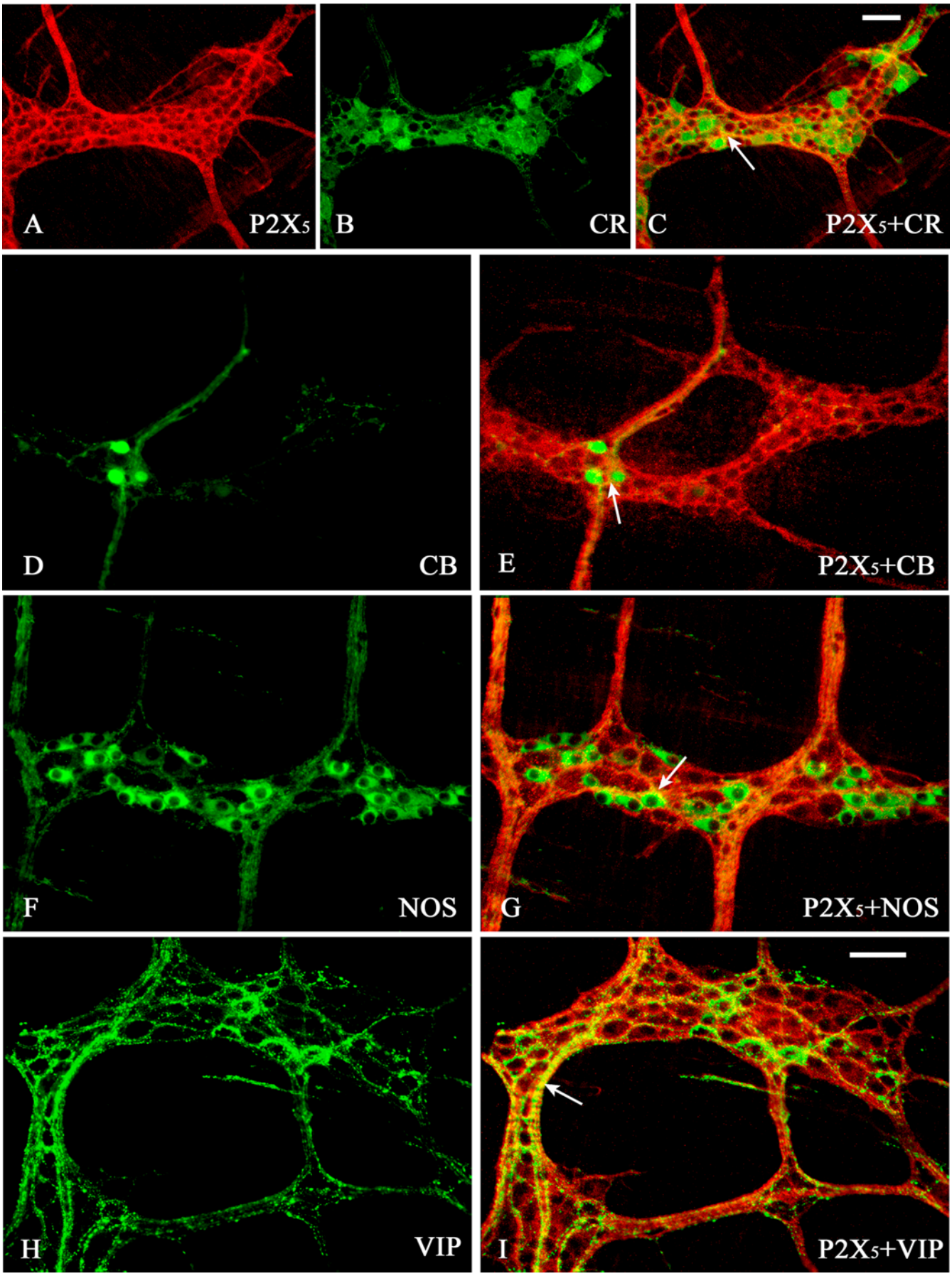


Fig. 2 Higher magnification examples of $P2X_5$ and $P2X_2$ receptor immunoreactivity in mouse enteric plexuses. **A** $P2X_5$ receptor immunoreactivity in the MP of the jejunum. Note that this is largely located in nerve fibres that envelope neurone cell bodies and possibly on glial cell processes. **B** $P2X_5$ receptor immunoreactivity in the SMPs of the colon. Note that this is located in neurone cell bodies and in nerve fibres. **C** $P2X_2$ receptor immunoreactivity in the MP of the ileum. Note labelling of both a nerve cell body and nerve fibres. *Bar* 25 μm


A
P2X5
B
CR
C
P2X5+CR
D
CB
E
P2X5+CB
F
NOS
G
P2X5+NOS
H
VIP
I
P2X5+VIP

◀ **Fig. 3** Colocalisation of $P2X_5$ receptor immunoreactivity in nerve fibres enveloping enteric nerves, with various neurochemical markers in the MP of mouse ileum. $P2X_5$ receptor immunoreactivity is labelled with Cy3 (*red*) and the neurochemical markers with FITC (*green*). $P2X_5$ receptor immunoreactivity was seen in nerve fibres surrounding ganglion cell soma with immunoreactivity for **A**–**C** calretinin (*CR*), **D**, **E** calbindin (*CB*) and **F**, **G** nitric oxide synthase (*NOS*). The nerve fibres with $P2X_5$ receptor immunoreactivity exhibited colocalisation with **H**, vasoactive intestinal peptide (*VIP*). Sites of rare colocalisation (*arrows*) appear *yellow*. *Bars* 50 μm

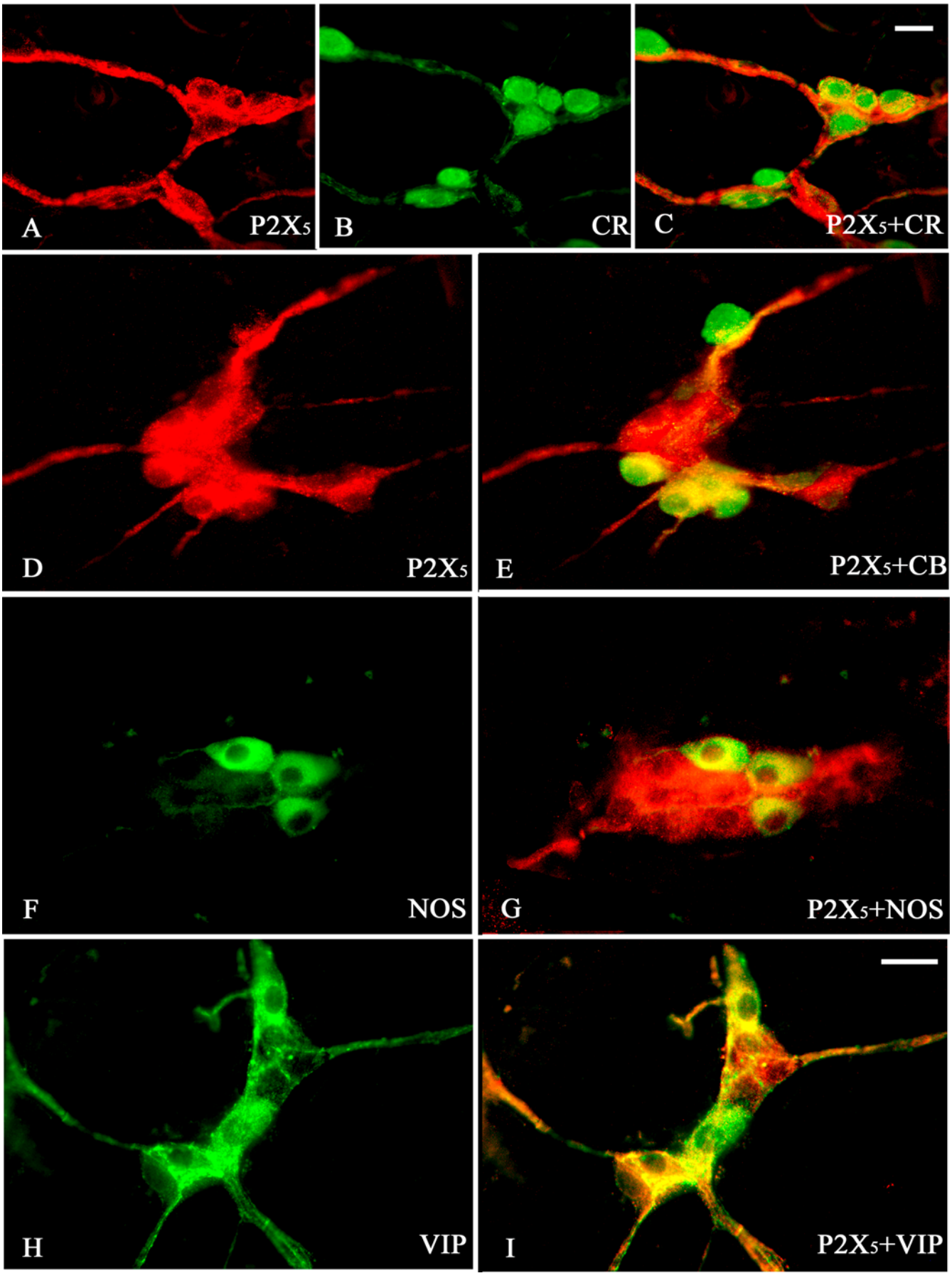
through P2X receptors (Barajas-López et al. 1996, 2000). Enteric neurones have been suggested predominantly to express $P2X_2$ receptors (Zhou and Galligan 1996; LePard et al. 1997), whereas a minority heterologously expresses $P2X_1$ and $P2X_3$ receptors (Zhou and Galligan 1996). Barajas-López et al. (1996) have observed that myenteric neurones express a P2X receptor showing pharmacological resemblances 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 neurones, suggesting that similar P2X receptors are present in both plexuses. The present study has shown that $P2X_2$ receptor immunoreactivity is widely distributed in both the MPs and SMPs throughout the entire length of the mouse digestive tract from the stomach to the colon. In the MPs, two kinds of $P2X_2$-positive ganglion neurones (strongly staining and weakly staining cells) have been seen. These results are consistent with previous studies of $P2X_2$ receptor localisation in guinea-pigs (Castelucci et al. 2002). The $P2X_2$ receptor is dominant but this does not preclude the possibility that other P2X receptor subtypes on enteric neurones also play important roles. Our study has also shown that $P2X_5$ receptor immunoreactivity is widely distributed along the whole length of the mouse gut. These observations of the distribution of $P2X_2$ and $P2X_5$ immunoreactivity in the small intestine are consistent with previous electrophysiological results (Zhou and Galligan 1996). Approximately 80% of cultured myenteric ganglion cells in guinea-pig intestine are reported to respond to the P2X receptor antagonist, PPADS, which inhibits 97% of the hexamethonum-resistant fast EPSPs. Zhou and Galligan (1996) claim that the electrophysiological properties of myenteric neurones in the small intestine indicate that 10% of them express $P2X_3$ or $P2X_1$ receptors, whereas most of them express $P2X_2$ or $P2X_5$ receptors.

To date, the evidence for the expression of four P2X receptor subtypes has been obtained in the ENS by electrophysiological or immunohistochemical methods. Immunoreactivity for $P2X_2$ (Castelucci et al. 2002; Giaroni et al. 2002), $P2X_3$ (Poole et al. 2002; Van Nassauw et al. 2002) and $P2X_7$ (Hu et al. 2001) receptors, in addition to our results for the $P2X_5$ receptor, has been reported. Neither $P2X_1$ nor $P2X_4$ receptor immunoreactivity has been found in cultured myenteric neurones (Zhou and Galligan 2000) or whole-mount preparations of enteric ganglia (unpublished observations).

In this study, we have demonstrated, for the first time, the presence of $P2X_5$ receptor immunoreactivity in enteric neurones of mouse. We have found abundant $P2X_5$ receptor immunoreactivity at the surfaces of ganglion cell bodies in MPs and in association with nerve fibres, although the possibility that $P2X_5$ receptors are also localised on glial cell processes cannot be excluded. The distribution of the receptor on the surfaces of cell bodies suggests that they could be activated by ATP released as a neurotransmitter or as a paracrine mediator released from other cellular sources. Double-labelling of the $P2X_5$ receptor in combination with immunoreactivity for calretinin, calbindin and NOS has revealed $P2X_5$ receptor immunoreactivity variably colocalised with neurones expressing these neurochemical markers. Many of the nerve fibres in the MP of ileum are immunoreactive for $P2X_5$ and for VIP. According to the chemical coding of the enteric neurone classes in the mouse small intestine (Sang and Young 1996), there are two major classes of circular muscle motor neurones: one class is characterised by the presence of NOS, VIP and neuropeptide Y (NPY), whereas the second class contains calretinin plus substance P. In the guinea-pig small intestine (Costa et al. 1996; Brookes 2001), the neurones exhibiting calretinin immunoreactivity can be identified as excitatory motor neurones and ascending interneurones, the neurones expressing NOS immunoreactivity are thought to be inhibitory motor neurones and descending interneurones and the neurones expressing calbindin immunoreactivity are thought to be primary afferent neurones. These findings suggest that specific roles are mediated via receptors containing $P2X_5$ subunits and that $P2X_5$ receptors could be heteromeric for other subunits, particularly $P2X_2$.

Table 2 Quantitative analysis of double-labelling studies for $P2X_5$ with calretinin, calbindin, neuronal nitric oxide synthase (*NOS*) and vasoactive intestinal peptide (*VIP*) in mouse ganglia of the ileum SMPs (*n* number of double-labelled cell profiles and the total number of cell profiles counted, respectively, for each combination of receptor)

Markers	$P2X_5$-immunoreactive neurones containing calretinin, calbindin, NOS or VIP	Calretinin-, calbindin-, NOS- or VIP-immunoreactive neurones containing $P2X_5$
Calretinin *n*	22±4% (437/1986)	91±3% (437/481)
Calbindin *n*	9±2% (169/1877)	86±5% (169/196)
NOS *n*	6±2% (117/1923)	96±1% (117/122)
VIP *n*	68±4% (1259/1851)	92±3% (1259/1368)


A
P2X5
B
CR
C
P2X5+CR
D
P2X5
E
P2X5+CB
F
NOS
G
P2X5+NOS
H
VIP
I
P2X5+VIP

◀ **Fig. 4** Higher magnification of the colocalisation (*yellow/orange*) of $P2X_5$ receptor immunoreactivity with various neurochemical markers in the SMP of mouse ileum. $P2X_5$ receptor immunoreactivity is labelled with Cy3 (*red*) and the neurochemical markers with FITC (*green*). $P2X_5$ receptor immunoreactivity was seen with immunoreactivity for **A**–**C** calretinin (*CR*), **D**, **E** calbindin (*CB*), **F**, **G** nitric oxide synthase (*NOS*), **H**, **I** vasoactive intestinal peptide (*VIP*). *Bars* 25 μm

In the SMP, $P2X_5$ receptor immunoreactivity occurs throughout the cytoplasm and on the surface membranes of the nerve cell bodies, nerve fibres and nerve bundles. Colocalisation of $P2X_5$ receptors with calretinin, calbindin, NOS and VIP in the SMP of mouse ileum has shown that 22%, 9%, 6% and 68% of neurones with $P2X_5$ receptor immunoreactivity are also immunoreactive for calretinin, calbindin, NOS and VIP, respectively. Earlier immunocytochemical studies have demonstrated that four classes of nerve cell bodies account for almost all neurones in the submucosal ganglia of the guinea-pig ileum (Furness et al. 1984; Quinson et al. 2001). These are (1) Dogiel type II neurones immunoreactive for choline acetyltransferase (ChAT), tachykinins and calbindin (about 10% of nerve cells), (2) neurones immunoreactive for calretinin and ChAT (about 15%), (3) neurones with VIP immunoreactivity (about 45%) and (4) neurones with both NPY and ChAT immunoreactivity (about 30%). Our results suggest that $P2X_5$ receptor immunoreactivity occurs at least on three classes of neurones in the submucosal ganglia of the mouse ileum.

In conclusion, abundant $P2X_5$ receptors are expressed in the MP and SMP of the mouse gastrointestinal tract. This subunit of P2X receptor may be specific to functional subtypes of enteric neurone. The $P2X_5$ receptor and other receptors may form heteromultimers that would have a unique pharmacology.

Acknowledgements The authors thank Dr. Chrystalla Orphanides for editorial assistance.

References

Abbracchio MP, Burnstock G (1994) Purinoceptors: are there families of P2X and P2Y purinoceptors? Pharmacol Therap 64:445–475

Barajas-López C, Huizinga JD, Collins SM, Gerzanich V, Espinosa-Luna R, Peres AL (1996) P2X-purinoceptors of myenteric neurones from the guinea-pig ileum and their unusual pharmacological properties. Br J Pharmacol 119:1541–1548

Barajas-López C, Espinosa-Luna R, Christofi FL (2000) Changes in intracellular Ca^{2+} by activation of P2 receptors in submucosal neurons in short-term cultures. Eur J Pharmacol 409:243–257

Belai A, Burnstock G (1994) Evidence for the coexistence of ATP and nitric oxide in non-adrenergic, non-cholinergic (NANC) inhibitory neurones in the rat ileum, colon and annococcygeus muscle. Cell Tissue Res 278:197–200

Bian X, Ren J, DeVries M, Schnegelsberg B, Cockayne DA, Ford AP, Galligan JJ (2003) Peristalsis is impaired in the small intestine of mice lacking the $P2X_3$ subunit. J Physiol (Lond) 551:309–322

Brandle U, Guenther E, Irrle C, Wheeler-Schilling TH (1998) Gene expression of the P2X receptors in the rat retina. Brain Res Mol Brain Res 59:269–272

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

Burnstock G (1978) A basis for distinguishing two types of purinergic receptor. In: Straub RW, Bolis L (eds) Cell membrane receptors for drugs and hormones: a multidisciplinary approach. Raven Press, New York, pp 107–118

Burnstock G (2001) 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 (2003) Purinergic receptors in the nervous system. In: Schwiebert EM (ed) Current topics in membranes. Purinergic receptors and signalling, vol 54. Academic Press, San Diego, pp 307–368

Burnstock G, Lavin S (2002) Interstitial cells of Cajal and purinergic signalling. Autonom Neurosci 97:68–72

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

Collo G, North RA, Kawashima E, Merlo-Pich E, Neidhart S, Surprenant A, Buell G (1996) Cloning of $P2X_5$ and $P2X_6$ receptors and the distribution and properties of an extended family of ATP-gated ion channels. J Neurosci 16:2495–2507

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

Crowe R, Burnstock G (1981) Comparative studies of quinacrine-positive neurons in the myenteric plexus of stomach and intestine of guinea-pig, rabbit and rat. Cell Tissue Res 221:93–107

De Man JG, De Winter BY, Seerden TC, De Schepper HU, Herman AG, Pelckmans PA (2003) Functional evidence that ATP or a related purine is an inhibitory NANC neurotransmitter in the mouse jejunum: study on the identity of P2X and P2Y purinoceptors involved. Br J Pharmacol 140:1108–1116

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

Furness JB, Costa M, Keast JR (1984) Choline acetyltransferase and peptide immunoreactivity of submucous neurons in the small intestine of the guinea-pig. Cell Tissue Res 237:329–336

Galligan JJ, Bertrand PP (1994) ATP mediates fast synaptic potentials in enteric neurons. J Neurosci 14:7563–7571

Galligan JJ, LePard KJ, Schneider DA, Zhou X (2000) Multiple mechanisms of fast excitatory synaptic transmission in the enteric nervous system. J Auton Nerv Syst 81:97–103

Giaroni C, Knight GE, Ruan HZ, Glass R, Bardini M, Lecchini S, Frigo G, Burnstock G (2002) P2 receptors in the murine gastrointestinal tract. Neuropharmacology 43:1313–1323

Glass R, Burnstock G (2001) Immunohistochemical identification of cells expressing ATP-gated cation channels (P2X receptors) in the adult rat thyroid. J Anat 198:569–579

Glushakov AV, Glushakova Y, Skok VI (1998) Two types of P2X-purinoceptors in neurons of the guinea pig ileum submucous plexus. Neurophysiology 30:301–304

Greig AVH, Linge C, Terenghi G, McGrouther DA, Burnstock G (2003) Purinergic receptors are part of a functional signalling system for proliferation and differentiation of human epidermal keratinocytes. J Invest Dermatol 120:1007–1015

Gröschel-Stewart U, Bardini M, Robson T, Burnstock G (1999a) P2X receptors in the rat duodenal villus. Cell Tissue Res 297:111–117

Gröschel-Stewart U, Bardini M, Robson T, Burnstock G (1999b) Localisation of $P2X_5$ and $P2X_7$ receptors by immunohistochemistry in rat stratified squamous epithelia. Cell Tissue Res 296:599–605

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

Johnson PJ, Shum OR, Thornton PD, Bornstein JC (1999) Evidence that inhibitory motor neurons of the guinea-pig small intestine exhibit fast excitatory synaptic potentials mediated via P2X receptors. Neurosci Lett 266:169–172

Khakh BS, Burnstock G, Kennedy C, King BF, North RA, Seguela 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

Lee H-Y, Bardini M, Burnstock G (2000) Distribution of P2X receptors in the urinary bladder and the ureter of the rat. J Urol 163:2002–2007

LePard KJ, Galligan JJ (1999) Analysis of fast synaptic pathways in myenteric plexus of guinea pig ileum. Am J Physiol 276:G529–G538

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

McConalogue K, Todorov L, Furness JB, Westfall DP (1996) Direct measurement of the release of ATP and its major metabolites from the nerve fibres of the guinea-pig taenia coli. Clin Exp Pharm Physiol 23:807–812

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

Poole DP, Castelucci P, Robbins HL, Chiocchetti R, Furness JB (2002) The distribution of $P2X_3$ purine receptor subunits in the guinea pig enteric nervous system. Auton Neurosci 101:39–47

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

Ralevic V, Burnstock G (1998) Receptors for purines and pyrimidines. Pharmacol Rev 50:413–492

Ren J, Bian X, DeVries M, Schnegelsberg B, Cockayne DA, Ford AP, Galligan JJ (2003) $P2X_2$ subunits contribute to fast synaptic excitation in myenteric neurons of the mouse small intestine. J Physiol (Lond) 552:809–821

Ryten M, Hoebertz A, Burnstock G (2001) Sequential expression of three receptor subtypes for extracellular ATP in developing rat skeletal muscle. Dev Dyn 221:331–341

Ryten M, Dunn PM, Neary JT, Burnstock G (2002) ATP regulates the differentiation of mammalian skeletal muscle by activation of a $P2X_5$ receptor on satellite cells. J Cell Biol 158:345–355

Sang Q, Young HM (1996) Chemical coding of neurons in the myenteric plexus and external muscle of the small and large intestine of the mouse. Cell Tissue Res 284:39–53

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

Van Nassauw L, Brouns I, Adriaensen D, Burnstock G, Timmermans JP (2002) Neurochemical identification of enteric neurons expressing $P2X_3$ receptors in the guinea-pig ileum. Histochem Cell Biol 118:193–203

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 (P2X receptors) determined by immunocytochemistry. Proc Natl Acad Sci USA 93:8063–8067

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

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 (Lond) 496:719–729

Zhou X, Galligan JJ (2000) Properties of the nicotinic cholinergic receptors and P2X receptors in myenteric neurons from guinea pig small intestine. Soc Neurosci Abstr 719–729:1633