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ORIGINAL PAPER

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$P2X_2$ and $P2X_3$ purinoceptors in the rat enteric nervous system

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Abstract Adenosine 5′-triphosphate receptors are known to be involved in fast excitatory postsynaptic currents in myenteric neurons of the digestive tract. In the present study, the distribution of $P2X_2$ and $P2X_3$ receptor mRNA was examined by in situ hybridisation while $P2X_2$ and $P2X_3$ receptor protein was localised by immunohistochemical methods. In addition, $P2X_2$ and $P2X_3$ receptors were colocalised with calbindin and calretinin in the myenteric and submucosal plexus. $P2X_2$- and $P2X_3$-immunoreactive neurons were found in the myenteric and submucosal plexuses throughout the entire length of the rat digestive tract from the stomach to the colon. Approximately 60%, 70% and 50% of the ganglion cells in the myenteric plexus of the gastric corpus, ileum and distal colon, and 56% and 45% in the submucosal plexus of the ileum and distal colon, respectively, showed positive immunoreactivity to the $P2X_2$ receptor. Approximately 10%, 2% and 15% of the ganglion cells in the myenteric plexus of the gastric corpus, ileum and distal colon, and 62% and 40% in the submucosal plexus of the ileum and distal colon, respectively, showed positive immunoreactivity to the $P2X_3$ receptor. Double-labelling studies showed that about 10–25% of the neurons with $P2X_2$ immunoreactivity in myenteric plexus and 30–50% in the submucosal plexus were found to express calbindin or calretinin. About 80% of the neurons with $P2X_3$ receptor immunoreactivity in the myenteric plexus and about 40% in the submucosal plexus expressed calretinin. Approximately 30–75% of the neurons with $P2X_3$ receptor immunoreactivity in the submucosal plexus expressed calbindin, while none of them were found to express calbindin in the myenteric plexus.

Keywords Enteric nervous system · $P2X_2$ · $P2X_3$ · Calbindin · Calretinin · Rat

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Introduction

Adenosine 5′-triphosphate (ATP) is established as a cotransmitter in the nervous system (Burnstock 1997). P2X receptors have been shown to play an important role in synaptic transmission within the neural pathways mediating motor behaviour in the intestine (Katayama and Morita 1989; Kimball et al. 1996; Heinemann et al. 1999; Bian et al. 2000; Spencer et al. 2000) and the noncholinergic portion of fast excitatory postsynaptic potentials are mediated by P2X receptors (Lepard et al. 1997; Zhou and Galligan 1998; see Burnstock 2001a). Seven P2X receptor subunits have been cloned and each P2X receptor subtype has defined pharmacological and electrophysiological properties and tissue distribution (Brake et al. 1995; Bo et al. 1995; Chen et al. 1995; Valera et al. 1995; Collo et al. 1996; Surprenant et al. 1996). Using immunohistochemical methods, Castelucci et al. (2002) described the distribution of $P2X_2$ purinoceptors in the guinea pig enteric nervous system, while enteric neurons expressing $P2X_3$ receptors in the guinea pig intestine have also been reported (Poole et al. 2002; Van Nassauw et al. 2002). These data showed that both $P2X_2$ and $P2X_3$ purinoceptor subunits are expressed on inhibitory motor neurons, noncholinergic secretomotor neurons, but only the $P2X_2$ subunit is expressed in the intrinsic primary afferent neurons in the guinea pig ileum. In the myenteric plexuses of human colon, $P2X_3$ subunits were found to coexist with calbindin D-28 k (Fajobi and Burnstock, unpublished data). Calbindin is believed to be a marker for the intrinsic sensory neurons in the guinea pig gut (see Furness et al. 1998). So far the data describing the expression pattern of P2X purinoceptors has focused on one species, namely the guinea pig. There

may be differences between species. In this study, we have used rats to compare the distribution pattern of both $P2X_2$ and $P2X_3$ receptor subunits in the submucosal and myenteric plexuses along the length of the rat gut and determine whether they coexist with calbindin D-28 k and calretinin, using immunocytochemistry and in situ hybridisation.

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 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. Ten Wistar rats (200–250 g) were killed by asphyxiation with CO_2 and perfused through the aorta with 100 ml 0.9% NaCl solution and 250 ml 4% paraformaldehyde in 0.1 mol/l phosphate buffer, pH 7.4. The digestive tracts were removed and washed with phosphate-buffered saline (PBS) and then refixed in 4% paraformaldehyde in 0.1 mol/l phosphate buffer, pH 7.4, overnight. The mucosa was discarded and the submucosal and myenteric plexuses of the gastric corpus, jejunum, ileum and distal colon of the rats were used as whole-mount preparations.

Immunocytochemistry

The development and specificity of the $P2X_2$ and $P2X_3$ polyclonal antisera have been reported previously (Xiang et al. 1998a; Oglesby et al. 1999). The immunocytochemical method was modified from our previous report (Xiang et al. 1998b). Briefly, the preparations were washed 3×5 min in 0.01 mol/l pH 7.2 PBS and then incubated in 1.0% H_2O_2 for 30 min to block the endogenous peroxidase. The preparations were preincubated in 10% normal horse serum (NHS), 0.2% Triton X-100 in PBS for 30 min, followed by incubation with $P2X_2$ or $P2X_3$ antisera, diluted 1:500 in antiserum 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, PA, USA) diluted 1:500 in antiserum dilution solution for 1 h at 37°C, and then with streptavidin-HRP (Sigma) diluted 1:1,000 in PBS for 1 h at 37°C. Finally, a nickel-intensified diaminobenzidine reaction was used to visualise immunoreactivity. All the incubations and reactions were separated by 3×10 min washes in PBS. The preparations were mounted, dehydrated, cleared and covered.

The following protocol was used for double staining of $P2X_2$ or $P2X_3$ with calbindin D-28 k or calretinin. The preparations were washed 3×5 min in PBS, and then preincubated in antiserum dilution solution for 30 min, followed by incubation with $P2X_2$ and $P2X_3$ antisera diluted 1:500, calbindin (mouse anti-rat; SWANT) diluted 1:5,000 and calretinin (mouse anti-rat; SWANT) diluted 1:2,000 in antiserum solution, overnight at 4°C. Subsequently, the preparations were incubated with Cy3-conjugated donkey anti-rabbit IgG (Jackson) diluted 1:300 for P2X antisera and FITC-conjugated donkey anti-mouse IgG (Jackson) diluted 1:200 for calbindin and calretinin in antiserum dilution solution for 1 h at room temperature. All the incubations and reactions were separated by 3×10 min washes in PBS.

Control experiments were carried out with $P2X_2$ and $P2X_3$ antisera preabsorbed with $P2X_2$ and $P2X_3$ peptides. No staining was observed in those preparations incubated with antiserum solutions preabsorbed with $P2X_2$ and $P2X_3$ peptides.

In situ hybridisation

Sense and antisense digoxigenin-labelled cRNA probes were synthesised with a DIG-RNA labelling kit (Boehringer Mannheim, Lewes, UK) using truncated $rP2X_2$ and $rP2X_3$ cDNAs as the templates. The cRNA sequences corresponded to the nucleotide sequences 1–994 in $rP2X_2$ cDNA (GenBank U14414) and 1–673 in $rP2X_3$ cDNA (GenBank X90651). In situ hybridisation was carried out using our previously described method (Xiang et al. 2001). Briefly, the whole-mount preparations were washed in 0.1 M glycine/PBS and in 0.4% Triton X-100/PBS for 10 min each. The preparations were then incubated in protease K (1 μg/ml) in PBS (Sigma) for 30 min at 37°C. The activity of protease K was stopped by fixation in 4% paraformaldehyde for 5 min, followed by a 2×3 min wash in PBS to remove the fixative from the preparations. The preparations were incubated in 0.25% acetic anhydride with 0.1 M triethanolamine (pH 8.0) for 10 min at room temperature, followed by washing in 2×saline sodium citrate (SSC) for 10 min. Digoxigenin-labelled cRNA (0.5 μg/ml) of either antisense or sense probe was added to the hybridisation solution containing 50% formamide, 10% dextran sulphate, 0.3 M NaCl, 1×Denhardt's solution, 0.05 M TRIS-HCl (pH 8.0), 1 mM EDTA and 250 μg/ml *E. coli* tRNA (RNase-free; Sigma). Hybridisation was carried out for 16 h at 63°C in a hybridisation oven. The preparations were washed in 4×SSC for 20 min at 37°C, followed by incubation in 2×SSC containing 20 μg/ml RNase A (Sigma) for 30 min at 37°C to digest the RNA probes that did not hybridise with the targeted RNA. The preparations were further washed in 1×SSC and 0.2×SSC at 37°C for 20 min, respectively.

The following method was used to detect hybridisation signals. Preparations were first incubated in blocking solution containing 5% bovine serum albumin and 0.4% Triton X-100 in PBS at room temperature for 30 min, and then with anti-digoxigenin antibody conjugated to alkaline phosphatase (Boehringer Mannheim) diluted 1:1,000 in blocking solution for 4 h at room temperature. The preparations were washed with PBS for 4×5 min, followed by washing in 0.1 M TRIS-HCl solution, pH 8.0, containing 0.1 M NaCl and 0.01 M $MgCl_2$, and then equilibrated for 10 min in 0.1 M TRIS-HCl solution, pH 9.5, with 0.1 M NaCl and 0.05 M $MgCl_2$. The colour development was performed with 400 μg/ml nitro blue tetrazolium, 200 µg/ml 5-bromo-4-chloro-3-indolyl phosphate and 100 μg/ml levamisole in 0.1 M TRIS-HCl solution, pH 9.5, containing 0.1 M NaCl and 0.05 M $MgCl_2$, in the dark, at room temperature, overnight. The preparations were rinsed in 10 mM TRIS-HCl, 1 mM EDTA, pH 8.0, for 10 min to stop the colour development, mounted with 50% glycerol in TRIS-HCl/EDTA solution and stored at 4°C in the dark.

Quantitative analysis

For single-label immunostaining and hybridisation, some of the preparations were counterstained with 1% neutral red after staining in order that the number of ganglion cells positive or negative for $P2X_2$ and $P2X_3$ could be calculated. Neurons were identified by their large and weakly stained nuclei. Some of the preparations were counterstained with PGP 9.5 antiserum in order to give another assessment of the number of neurons in the ganglia. Ten random fields at 200-fold magnification for one preparation of a gut segment were chosen to count the positive and negative neurons. Ten fields for each of three preparations from each of four rats were used for each marker. The mean from each rat was calculated and the values obtained from each rat averaged and the standard error of the mean calculated. Values for both the total number and percentage of neurons positive for a marker against total neurons were obtained.

For fluorescence immunocytochemistry, whole-mount preparations were used to perform a quantitative analysis as described previously (Van Nassauw et al. 2002). Briefly, positively stained neuron bodies in the submucosal and myenteric ganglia were counted per visual field (area of 0.62 mm^2). Ten randomly chosen fields in each whole-mount preparation and three preparations of

each rat were analysed. At least four rats were used for each marker. The percentage of neurons immunoreactive for a particular marker that were also positive for a different marker was calculated and expressed as mean ± standard error of the mean (*n*=number of rats used).

Results

Localisation of $P2X_2$ receptor immunoreactivity

Myenteric plexus

$P2X_2$ receptor immunoreactivity was found in the myenteric plexuses throughout the entire length of the rat digestive tract from the stomach to the colon. In the gastric corpus myenteric plexuses, about 60% of ganglion cells were positively-stained with the $P2X_2$ antiserum and two types of positive ganglion neurons, strongly- and weakly-staining nerve cells, were present. Positive staining was seen in the cytoplasm of strongly-staining cells, while only the area near the cytoplasm membrane of weakly-stained cells was positive (Fig. 1A). In the small intestine myenteric plexuses of whole-mount preparations, $P2X_2$-immunoreactive (ir) ganglion neurons were found in all ganglia and about 70% of ganglion cells were positively-immunostained for the $P2X_2$ antibody. About 80% of these stained intensely, while 20% of them were weakly-labelled (Fig. 1B). In the myenteric plexuses of distal colon, about 50% of ganglion neurons were immunostained intensely by the $P2X_2$ antiserum and the positive reaction products were precipitated in the cytoplasm (Fig. 1C). In some ganglionic areas, along the whole length of gut (including the stomach and all segments of the small intestine and colon) many $P2X_2$-ir fibres with varicose-like structures were present (Fig. 1A–C).

Submucosal plexus

$P2X_2$-ir neurons were seen in the submucosal plexuses of all the gastrointestinal segments examined. In most of these neurons, the staining was intense. The positive staining was seen in the cytoplasm of the cell bodies and no positive staining was found in the nuclei. Approximately 56% and 45% of ganglion neurons were $P2X_2$-ir in the submucosal plexus of ileum and distal colon, respectively. Bundles of fibres were also stained with the $P2X_2$ antibody in all the segments (Fig. 2D, G, J).

Localisation of $P2X_3$ receptor immunoreactivity

Myenteric plexus

Positive staining for the $P2X_3$ receptor was seen in the myenteric plexuses throughout the entire length of the rat digestive tract, although the number of positive cells was much fewer than those positive for the $P2X_2$ receptor. In the gastric corpus myenteric plexus, about 10% of ganglion cells were stained with the $P2X_3$ antiserum and two kinds of positive ganglion neurons, strongly- and weakly-staining cells, were also observed. Positive products were demonstrated in the cytoplasm of both strongly- and weakly-stained cells. Strongly-staining fibres were also present in the stomach myenteric plexus region (Fig. 1D). In the myenteric plexuses of all three segments of small intestine, only about 2% of ganglion neurons were shown to be $P2X_3$ receptor-positive. Most of these were strongly stained (Fig. 1E). In the distal colon, about 15% of ganglion neurons were immunostained by the $P2X_3$ antiserum and the positive reaction products were precipitated in the whole cytoplasm (Fig. 1F).

Submucosal plexus

$P2X_3$-ir neurons were seen in the submucosal plexuses of all segments. Most of these were moderately stained. The positive staining is in the cytoplasm of the cell bodies and no positive staining is found in the nuclei. About 62% and 40% of the ganglion neurons were $P2X_3$ receptor-positive in the submucosal plexus of ileum and distal colon, respectively (Table 1; Fig. 3A, D, G, J). Few fibres were positively-stained for the $P2X_3$ receptor in the submucosal plexuses of all segments of the gut studied. Table 1 summarises the percentages of $P2X_2$ and $P2X_3$ receptor-positive neurons in myenteric and submucosal plexuses of ileum and distal colon.

Table 1 Numbers and percentages of $P2X_2$ and $P2X_3$ receptor-immunoreactive neurons (by immunohistochemistry) in the myenteric (*MP*) and submucosal plexus (*SMP*) of gastric corpus, ileum and distal colon

Region	$P2X_2$		$P2X_3$	
	Mean number/whole mount	Percentage	Mean number/whole mount	Percentage
Stomach MP	244±32	60±8	46±14	10±3
Ileum MP	315±23	70±5	10±5	2±1
Ileum SMP	263±38	56±8	285±41	62±9
Distal colon MP	235±19	50±4	75±25	15±5
Distal colon SMP	203±18	45±4	192±29	40±6

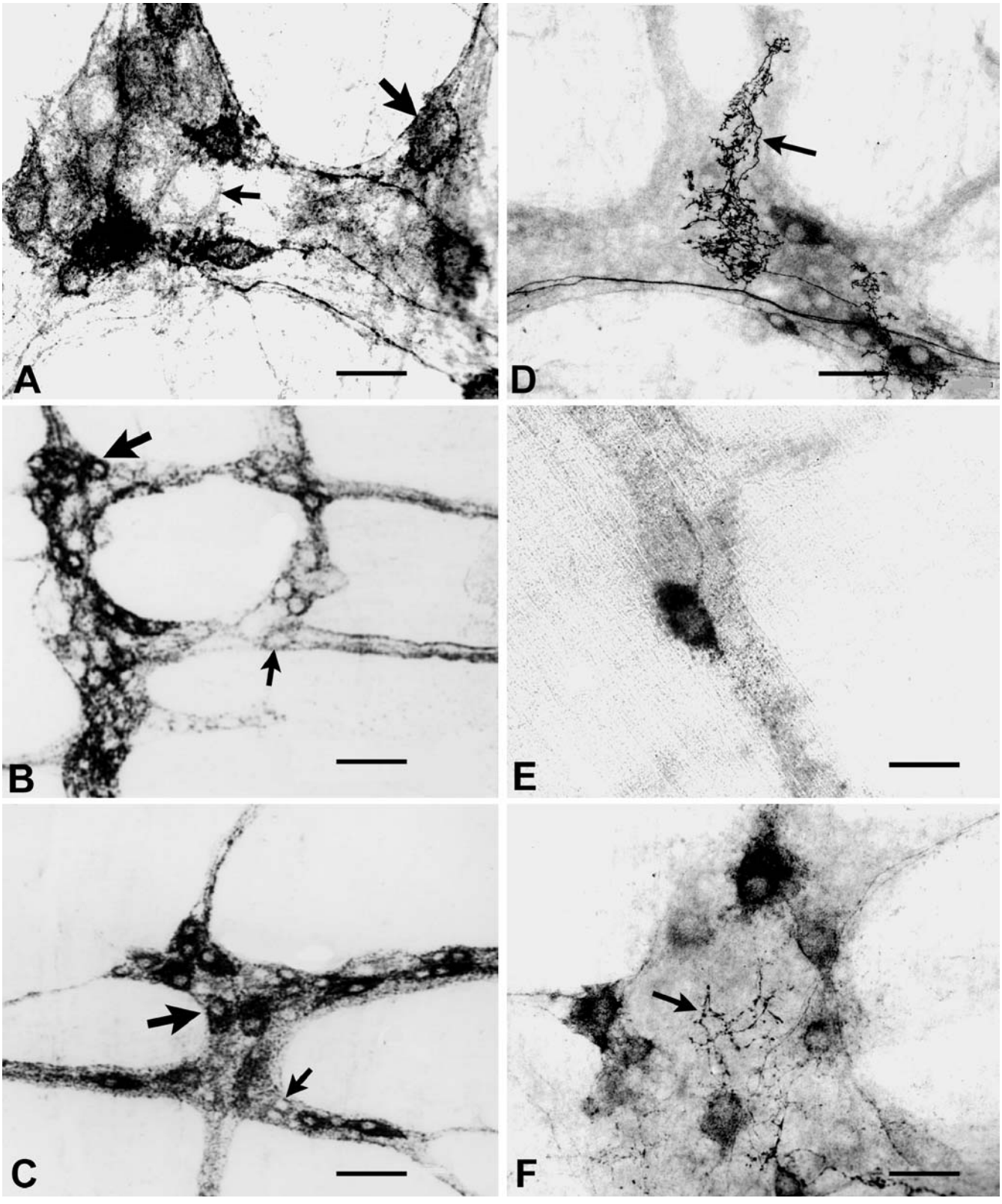

Fig. 1A–F $P2X_2$ and $P2X_3$ receptor-immunoreactive (-ir) neurons and nerve fibres in the rat myenteric plexus. Both intensely staining neurons (*thick arrows*) and weakly staining neurons (*thin arrows*) were present. **A** $P2X_2$ receptor-ir neurons and nerve fibres in the gastric corpus. **B** $P2X_2$ receptor-ir neurons and nerve fibres in the ileum. **C** $P2X_2$ receptor-ir neurons and nerve fibres in the distal colon. **D** $P2X_3$ receptor-ir neurons and nerve fibres in the gastric corpus. **E** A $P2X_3$ receptor-ir neuron and nerve fibre in the ileum. **F** $P2X_3$ receptor-ir neurons and nerve fibres in the distal colon. *Scale bars* 50 μm in **A**, **D–F**; 80 μm in **B**, **C**

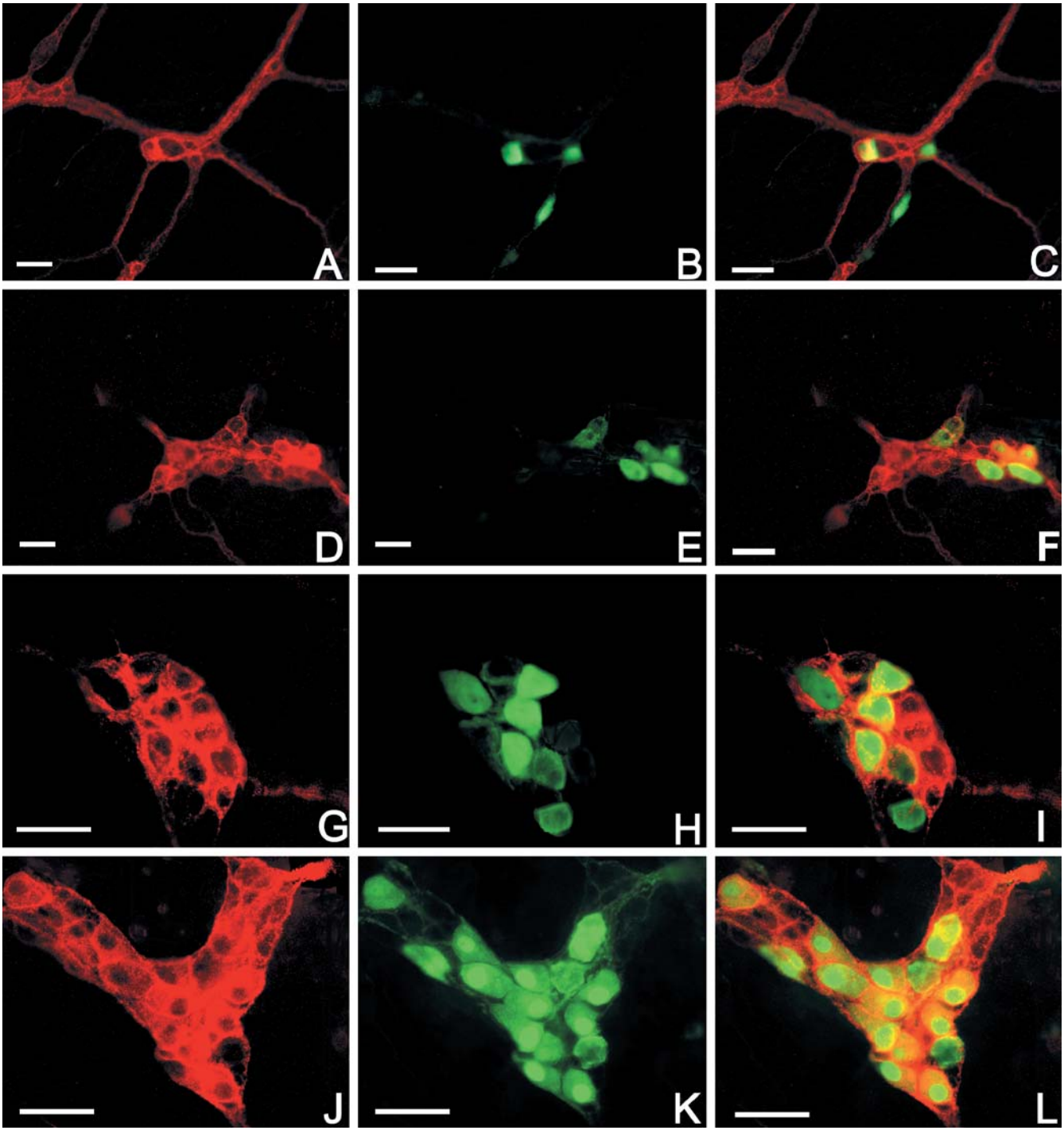

Fig. 2A–L Double immunolabelling with antisera directed against the $P2X_2$ receptor, calbindin and calretinin in the myenteric and submucosal plexuses of rat small intestine and colon. **A–C** Coexpression of $P2X_2$ and calretinin in the myenteric plexus of ileum. **D–F** Coexpression of $P2X_2$ and calbindin in the submucosal plexus of ileum. **G–I** Coexpression of $P2X_2$ and calretinin in the submucosal plexus of distal colon. **J–L** Coexpression of $P2X_2$ and calbindin in the submucosal plexus of distal colon. *Scale bars* 50 μm

Localisation of $P2X_2$ and $P2X_3$ receptor mRNAs

The distribution pattern of $P2X_2$ and $P2X_3$ receptor mRNA hybridisation signals was found to be similar to that of their immunoreactivity. The numbers and percentages which displayed $P2X_2$ or $P2X_3$ receptor mRNA hybridisation signals in the myenteric plexus of gastric corpus, ileum and distal colon, and submucosal plexuses of rat ileum and distal colon were found to be similar to those of $P2X_2$-ir or $P2X_3$-ir neurons. Table 2 summarises

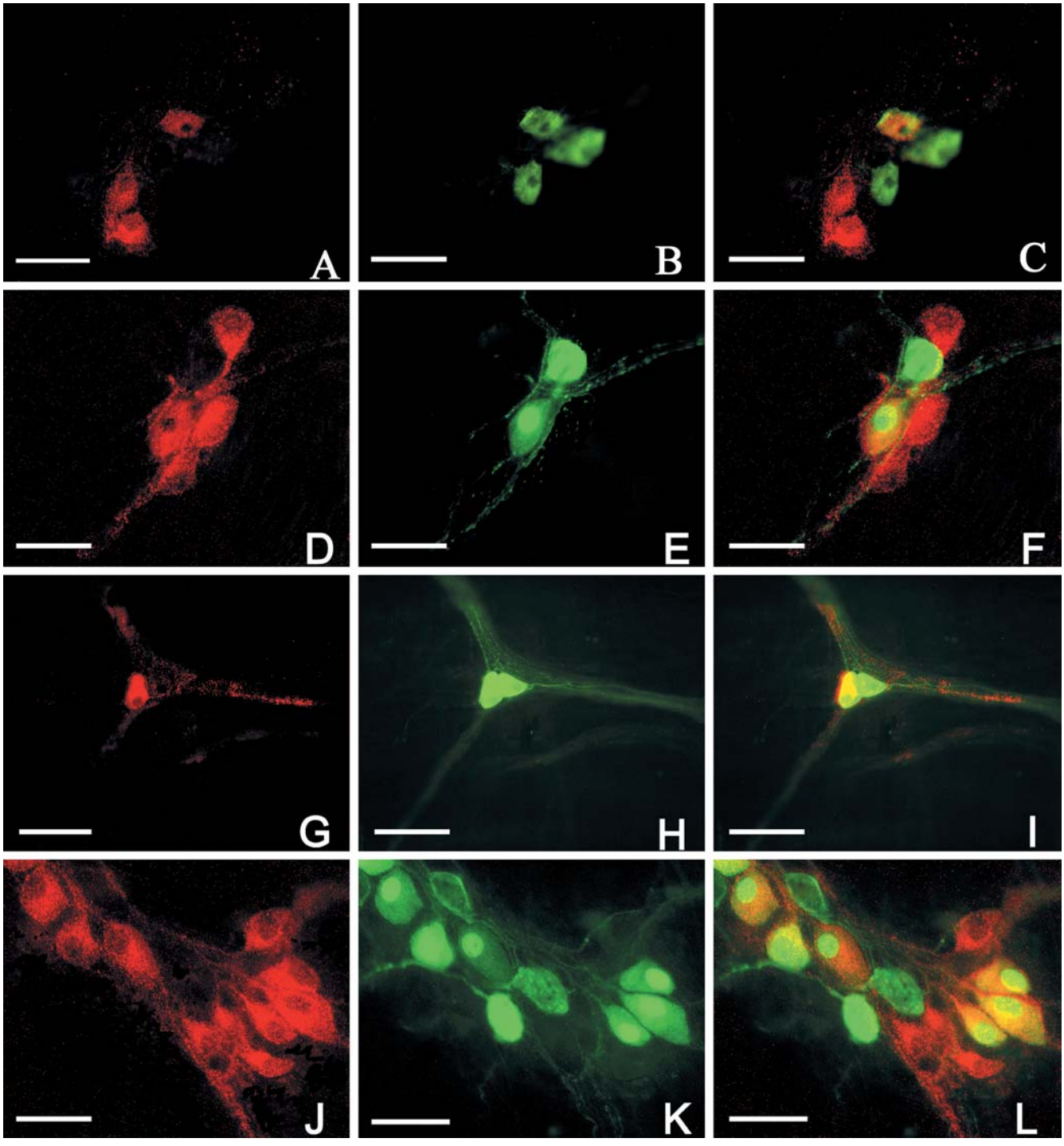

Fig. 3A–L Double immunolabelling with antisera directed against the $P2X_3$ receptor, calbindin and calretinin in the submucosal plexuses of rat small intestine and colon. **A–C** Coexpression of $P2X_3$ and calretinin in the submucosal plexus of ileum. **D–F** Coexpression of $P2X_3$ and calbindin in the submucosal plexus of ileum. **G–I** Coexpression of $P2X_3$ and calretinin in the submucosal plexus of distal colon. **J–L** Coexpression of $P2X_3$ and calbindin in the submucosal plexus of distal colon. *Scale bars* 50 μm

the percentages of $P2X_2$ and $P2X_3$ receptor mRNA-positive neurons in the myenteric plexuses of stomach, small intestine and colon, and submucosal plexuses of ileum and distal colon (Fig. 4A–H).

Double-labelling study

In the myenteric plexuses of small intestine and colon, no $P2X_3$-ir neurons were immunoreactive for calbindin D-28, although some $P2X_2$-ir neurons were immunoreactive for

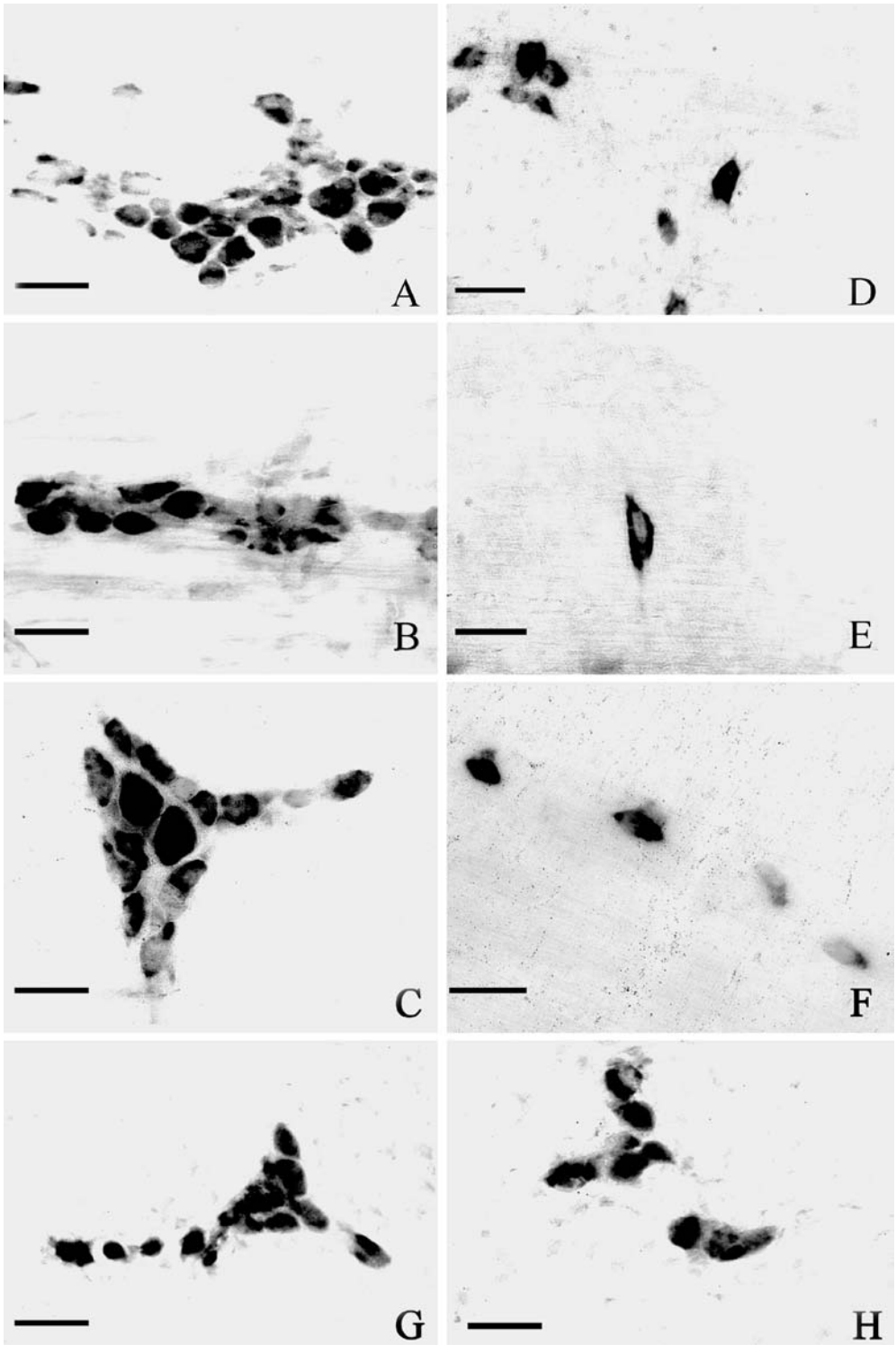

Fig. 4A–H $P2X_2$ and $P2X_3$ receptor mRNA-positive neurons (in situ hybridisation) in the rat myenteric and submucosal plexus. **A–C** $P2X_2$ receptor mRNA-positive neurons in the myenteric plexus of gastric corpus, ileum and distal colon, respectively. **D–F** $P2X_3$ receptor mRNA-positive neurons in the myenteric plexus of gastric corpus, ileum and distal colon, respectively. **G**, **H** $P2X_2$ and $P2X_3$ receptor mRNA-positive neurons, respectively, in the submucosal plexus of ileum. *Scale bars* 50 μm

calbindin. The majority of calbindin D-28-ir neurons showed weak immunoreactivity for $P2X_2$ receptors although there were also calbindin D-28-ir neurons with strong $P2X_2$ immunoreactivity (Fig. 2A–C). In the submucosal plexuses of the ileum and distal colon both $P2X_2$-ir and $P2X_3$-ir neurons were found to display calbindin D-28 (Figs. 2D–F, J–L, 3D–F, J–L), while calretinin-ir positive neurons in both myenteric and submucosal plexuses of ileum and distal colon were found to display $P2X_2$ or $P2X_3$ immunoreactivity (Figs. 2A–C, G–I, 3A–C, G–I). Tables 3 and 4 show the results of the quantitative analysis of double-labelling studies among $P2X_2$, $P2X_3$, calbindin and calretinin in the myenteric plexuses and submucosal plexuses.

Table 2 Numbers and percentages of $P2X_2$ and $P2X_3$ receptor mRNA-positive neurons (by in situ hybridisation) in the myenteric (*MP*) of gastric corpus, ileum and distal colon, and submucosal plexus (*SMP*) of ileum and distal colon

Region	$P2X_2$		$P2X_3$	
	Mean number/whole mount	Percentage	Mean number/whole mount	Percentage
Stomach MP	286±36	56±7	64±21	12±4
Ileum MP	360±29	75±6	18±12	3±2
Ileum SMP	279±32	62±7	312±55	71±8
Distal colon MP	260±30	52±6	94±21	18±4
Distal colon SMP	243±37	46±5	259±26	49±5

Table 3 Results of the quantitative analysis of double-labelling studies among $P2X_2$, calbindin (*CB*) and calretinin (*CR*) in the myenteric plexus (*MP*) and submucosal plexus (*SMP*) of ileum and distal colon. Both the numbers of neurons showing double labelling (for example, $P2X_2\text{-}ir^+$) and those not showing double labelling (for example, $P2X_2\text{-}ir^-$) are given and below them the percentages

Region	$P2X_2$-ir$^+$	$P2X_2$-ir$^+$	CB-ir$^+$	CB-ir$^+$	$P2X_2$-ir$^+$	$P2X_2$-ir$^+$	CR-ir$^+$	CR-ir$^+$
	CB-ir$^+$	CB-ir$^-$	$P2X_2$-ir$^+$	$P2X_2$-ir$^-$	CR-ir$^+$	CR-ir$^-$	$P2X_2$-ir$^+$	$P2X_2$-ir$^-$
Ileum MP	26±13	234+11	26±13	5±3	58±16	174±16	58±16	46±12
	10±5%	90±5%	83±7%	17±7%	25±7%	75±7%	56±5%	44±5%
Ileum SMP	81±18	173±13	81±18	23±6	102±22	146±19	102±22	29±7
	32±7%	68±7%	78±9%	22±9%	41±9%	59±9%	78±6%	22±6%
Distal colon MP	27±10	199±23	27±10	4±2	52±8	210±16	52±8	44±9
	12±6%	88±6%	89±7%	11±7%	20±8%	80±8%	54±6%	46±6%
Distal colon SMP	76±10	140±15	76±10	13±4	107±14	125±13	107±14	22±6
	35±8%	65±8%	85±7%	15±7%	46±6%	54±6%	83±8%	17±8%

Table 4 Results of the quantitative analysis of double labelling studies among $P2X_3$, calbindin (*CB*) and calretinin (*CR*) in the myenteric plexus (*MP*) and submucosal plexus (*SMP*) of ileum and distal colon. Both the numbers of neurons showing double labelling (for example, $P2X_3\text{-}ir^+$) and those not showing double labelling (for example, $P2X_3\text{-}ir^-$) are given and below them the percentages

Region	$P2X_3$-ir$^+$	$P2X_3$-ir$^+$	CB-ir$^+$	CB-ir$^+$	$P2X_3$-ir$^+$	$P2X_3$-ir$^+$	CR-ir$^+$	CR-ir$^+$
	CB-ir$^+$	CB-ir$^-$	$P2X_3$-ir$^+$	$P2X_3$-ir$^-$	CR-ir$^+$	CR-ir$^-$	$P2X_3$-ir$^+$	$P2X_3$-ir$^-$
Ileum MP	0	32±5	0	45±7	26±5	6±3	26±5	162±15
	0%	100%	0%	100%	81±9%	11±9%	16±5%	84±5%
Ileum SMP	79±15	185±26	79±15	104±16	95±13	165±18	95±13	37±6
	30±6%	70±6%	76±8%	24±8%	37±5%	73±5%	72±8%	28±8%
Distal colon MP	0	86±8	0	38±5	53±7	13±3	53±7	130±21
	0%	100%	0%	100%	83±7%	17±7%	29±5%	71±5%
Distal colon SMP	140±18	40±6	140±18	26±8	101±14	141±10	101±14	31±6
	75±10%	25±10%	79±10%	21±10%	40±5%	70±5%	76±7%	24±7%

Discussion

Exogenous and endogenous ATP, released during increase in intraluminal pressure, modifies intestinal peristalsis via different apamin-sensitive purine receptor mechanisms (Heinemann et al. 1999). Exogenous ATP depresses peristalsis mostly via suramin- and pyridoxalphosphate-6-azophenyl-3′,4′disulphonic acid (PPADS)-insensitive P2 receptors, whereas endogenous purines act via P2 receptors sensitive to both suramin and PPADS. A preliminary report claims that purinergic transmission is involved in a descending excitatory reflex in small intestine (see Bertrand 2003). P2X purinoceptors also play a significant role in transmission from descending interneurons to inhibitory motor neurons of the descending inhibitory reflex pathways excited by distension and mucosal distortion. However, at least two other forms of excitatory transmission to the inhibitory motor neurons can be identified. One of these is via nicotinic ACh receptors and the other is via P2 receptors (Bian et al. 2000). Evidence has been presented recently that ATP plays a major role in excitatory neuro-neuronal transmission in both ascending and descending reflex pathways to the longitudinal and circular muscles of the ileum, triggered by mucosal stimulation (Spencer et al. 2000), but P2X receptors have been reported to have no role in descending inhibitory reflexes in rat colon (Bian et al. 2003a). In a hypothesis presented by Burnstock (2001b), it was proposed that ATP, released from mucosal epithelial cells during distension, stimulated $P2X_3$ or $P2X_{2/3}$ receptors on low threshold subepithelial intrinsic sensory nerve fibres to initiate peristalsis, while high threshold extrinsic sensory nerve fibres mediated nociception. The mucosal processes of intrinsic sensory neurons in the guinea pig small intestine generate APs in response to ATP acting via excitatory P2X receptors. ATP released

within the mucosa may, thus, initiate or enhance reflexes in the guinea pig small intestine; ATP may be a sensory mediator in the guinea pig ileum and in other gastrointestinal tissues (Bertrand and Bornstein 2002). These data supported Burnstock's hypothesis of purinergic mechanosensory transduction in the gut (Burnstock 2001b; Wynn et al. 2003).

The present study shows that $P2X_2$ and $P2X_3$ receptor immunoreactivity is widely distributed in the whole length of the rat gut. Our findings regarding the distribution of $P2X_2$ and $P2X_3$ immunoreactivity in the small intestine are consistent with previous electrophysiological results obtained in intestine, where it was reported that about 80% of cultured myenteric ganglion cells responded to ATP, but were not antagonised by PPADS, suggesting that the $P2X_4$ receptor might be involved (Zhou and Galligan 1996, 1998).

Seven P2X receptor subunits have been cloned (Brake et al. 1995; Bo et al. 1995; Chen et al. 1995; Valera et al. 1995; Collo et al. 1996; Surprenant et al. 1996). There has been some ambiguity in the interpretation of electrophysiological results regarding which P2X receptor subunit(s) are present in myenteric ganglion neurons. Zhou and Galligan (1996) claimed that the electrophysiological properties of myenteric neurons in the small intestine indicated that 10% of them expressed $P2X_1$ or $P2X_3$ receptors and that most of them expressed $P2X_2$ or $P2X_5$ receptors. The report of Lepard et al. (1997) showed that the $P2X_2$ receptor was the main purinoceptor subunit, but Barajas-Lopez et al. (1996) claimed that $P2X_4$ and $P2X_6$, perhaps as heteromultimers, were dominantly expressed on myenteric neurons. The present results are consistent with the conclusion of Lepard et al. (1997) and Zhou and Galligan (1996).

The low level of immunoreactivity (about 20%) of the neurons staining for the $P2X_2$ receptor may indicate that these neurons possess heteromultimers of $P2X_2$ receptors, together with $P2X_3$ receptor subunits. Heteromultimers for $P2X_{2/3}$ (Lewis Neidhart et al. 1995; Liu et al. 2001), $P2X_{4/6}$ (Lê et al. 1998) and $P2X_{2/6}$ (King et al. 2000) have been previously described and $P2X_{2/3}$ heteromultimers have been reported on sensory nerves in bladder (Loesch et al. 2002; Rong et al. 2002; Zhong et al. 2003).

Previous morphological and functional data have shown that the P2X receptor subunits $P2X_1$, $P2X_2$, $P2X_4$ and $P2X_6$ are predominant in the central nervous system (Collo et al. 1996; Loesch and Burnstock 1998; Xiang et al. 1998b; Kanjhan et al. 1999; Loesch et al. 1999; Dunn et al. 2001) and in autonomic and sensory ganglia (Zhong et al. 1998, 2000a, b). In the enteric nervous system we have shown that the $P2X_2$ receptor is dominant but this does not preclude the possibility that other P2X receptors also play important roles in the myenteric ganglia.

Previous reports on purinoceptors in the gut focused on the intestine, particularly the ileum, but rarely on the stomach, except for the report by Lepard et al. (1997), which claimed that the myenteric ganglion cells in the gastric corpus expressed few if any, P2X receptors. In contrast, our results showed that 60% and 10% of the ganglion neurons in myenteric plexus of the stomach of rat expressed the $P2X_2$ and $P2X_3$ receptor subunits, respectively, and that most of these were stained intensely. Species differences might account for this discrepancy.

In the myenteric plexus of the colon, the distribution pattern of positive $P2X_2$ receptor immunostaining showed some differences from that of small intestine myenteric plexus, but was similar to that of the gastric myenteric plexus. It is therefore possible that the pharmacological and electrophysiological responses to purines of some of the myenteric ganglion neurons in the stomach will show similarities with those of those ganglion neurons in the colon.

In this study, we showed that $P2X_3$-ir neurons in the submucosal plexus also displayed calbindin D-28, although this was not the case in the myenteric plexus. It is believed that calbindin is a marker for intrinsic primary afferent (intrinsic sensory) neurons in the guinea pig ileum (Furness et al. 1998). In the rat ileum, $P2X_3$ receptors were found not to coexpress with calbindin D-28 (Poole et al. 2002; Van Nassauw et al. 2002). In contrast, in the present study of the rat gut, we have shown that a subpopulation of submucosal neurons show colocalisation of $P2X_3$ receptors and calbindin, suggesting that $P2X_3$ receptors may be localised on intrinsic primary afferent neurons. Colocalisation of $P2X_3$ receptors and calbindin have also been reported recently in the rat colorectum (Wynn et al. 2003) and also in mouse where the use of a $P2X_3$ knockout mouse showed that $P2X_3$ receptors on AH (intrinsic sensory) enteric neurons participate in the neural pathways underlying peristalsis (Bian et al. 2003b). There are clearly species differences for $P2X_3$ expression in the intrinsic sensory neurons of gut, but it appears that in the rat intestine at least, there is support for the hypothesis of Burnstock (2001b). In both submucosal and myenteric plexuses, $P2X_2$-positive neurons were found to display calbindin D-28. In this study $P2X_2$- and $P2X_3$-positive neurons in submucosal and myenteric plexuses were shown to display calretinin, which is believed to be a marker for cholinergic secretomotor and vasomotor neurons (Li and Furness 1998). However, a previous study by Castelucci et al. (2002) reported that $P2X_2$ receptors did not coexpress with calretinin in the gut intestine. This appears to be another example of species differences.

In summary, using immunocytochemistry and in situ hybridisation, the present study has shown that $P2X_2$ purinoceptor subunits are distributed widely in the myenteric and submucosal plexuses of rat gastrointestinal tract, while $P2X_3$ purinoceptor subunits are present in only a small subpopulation of myenteric ganglionic neurons. However, a larger number of submucosal ganglionic neurons show positive staining for $P2X_3$ receptors, although the density of immunoreactivity for $P2X_3$ was lower than that for $P2X_2$. $P2X_2$ receptors coexpress with calbindin and calretinin in both submucosal and myenteric plexuses. $P2X_3$ receptors are coexpressed with calretinin in submucosal and myenteric plexuses and

with calbindin in the submucosal plexus, but not in myenteric plexus.

References

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

Bertrand PP (2003) ATP and sensory transduction in the enteric nervous system. Neuroscientist 9:243–260

Bertrand PP, Bornstein JC (2002) ATP as a putative sensory mediator: activation of intrinsic sensory neurons of the myenteric plexus via P2X receptors. J Neurosci 22:4767–4775

Bian XC, 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

Bian XC, Bornstein JC, Bertrand PP (2003a) Nicotinic transmission at functionally distinct synapses in descending reflex pathways of the rat colon. Neurogastroenterol Motil 15:161–171

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

Bo X, Zhang Y, Nassar M, Burnstock G, Schoepfer R (1995) A P2X purinoceptor cDNA conferring a novel pharmacological profile. FEBS Letts 375:129–133

Brake AJ, Wagenbach MJ, Julius D (1995) New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. Nature 317:519–523

Burnstock G (1997) The past, present and future of purine nucleotides as signaling molecules. Neuropharmacology 36:1127–1139

Burnstock G (2001a) Purinergic signalling in gut. In: Abbracchio MP, Williams M (eds) Handbook of experimental pharmacology, vol 151/I. Purinergic and pyrimidinergic signalling. Springer, Berlin Heidelberg New York, pp 141–238

Burnstock G (2001b) Purine-mediated signalling in pain and visceral perception. Trends in Pharmacol Sci 22:82–188

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

Chen CC, Akoplan AN, Sivilotti L, Colquhoun D, Burnstock G, Wood JN (1995) A P2X purinoceptor expressed by a subset of sensory neurons. Nature 277:428–431

Collo G, North RA, Kawashima E, MerloPich 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

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

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

Heinemann A, Shahbazian A, Bartho L, Holzer P (1999) Different receptors mediating the inhibitory action of exogenous ATP and endogenously released purines on intestinal peristalsis. Br J Pharmacol 128:313–320

Kanjhan R, Houseley GD, Burton LD, Christie DL, Kippenberger Athorne PR, Luo L, Ryan AF (1999) Distribution of the $P2X_2$ receptor subunit of the ATP-gated ion channels in the rat central nervous system. J Comp Neurol 407:11–32

Katayama Y, Morita K (1989) Adenosine 5′-triphosphate modulates membrane potassium conductance in myenteric neurons. J Physiol 408:373–390

Kimball BC, Yule DI, Mulholland MW (1996) Extracellular ATP mediates Ca^{2+} signaling in cultured myenteric neurons via a PLC-dependent mechanism. Am J Physiol 270:G587–G593

King BF, Townsend-Nicholson A, Wildman SS, Thomas T, Spyer KM, Burnstock G (2000) Coexpression of rat $P2X_2$ and $P2X_6$ subunits in *Xenopus* oocytes. J Neurosci 20:4871–4877

Lê KT, Babinski K, Seguela P (1998) Central P2X4 and P2X6 channel subunits coassemble into a novel heteromeric ATP receptor. J Neurosci 18:7152–7159

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

Lewis Neidhart S, Holy C, North RA, Buell G, Surprenant A (1995) Co-expression of $P2X_2$ and $P2X_3$ receptor subunits can account for ATP-gated currents in sensory neurons. Nature 377:432–435

Li ZS, Furness JB (1998) Immunohistochemical localization of cholinergic markers in putative intrinsic primary afferent neurons of the small intestine. Cell Tissue Res 204:35–43

Liu M, King BF, Dunn PM, Rong W, Townsend-Nicholson A, Burnstock G (2001) Coexpression of $P2X_3$ and $P2X_2$ receptor subunits in varying amounts generates heterogeneous populations of P2X receptors that evoke a spectrum of agonist responses comparable to that seen in sensory neurons. J Pharmacol Exp Ther 296:1043–1050

Loesch A, Burnstock G (1998) Electron-immunocytochemical localization of the $P2X_1$ receptor in the rat cerebellum. Cell Tissue Res 294:253–260

Loesch A, Miah S, Burnstock G (1999) Ultrastructural localization of ATP-gated $P2X_2$ receptor immunoreactivity in the rat hypothalamo-neurohypophysial system. J Neurocytol 28:495–504

Loesch A, Glass R, Burnstock G (2002) Ultrastructural identification of $P2Y_2$ receptor mRNA in the rat thymus. Cells Tissues Organs 172:255–264

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

Rong W, Spyer M, Burnstock G (2002) Activation and sensitisation of low and high threshold afferent fibres mediated by P2X receptors in the mouse urinary bladder. J Physiol 541:591–600

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

Surprenant A, Rassendern F, Kawshima E, North RA, Buell G (1996) The cytolytic P2z receptor of extracelluar ATP identified as a P2X receptor ($P2X_7$). Science 272:735–738

Valera S, Hussy N, Evans RJ, Adani N, North AR, Surprenant A, Buell G (1995) A new class of ligand-gated ion channels defined by P2X receptor for extracellular ATP. Nature 371:516–519

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

Wynn G, Rong W, Xiang Z, Burnstock G (2003) Purinergic mechanisms contribute to mechanosensory transduction in the rat colorectum. Gastroenterology 125:1398–1409

Xiang Z, Bo X, Burnstock G (1998a) P2X receptor immunoreactivity in the rat cochlea, vestibular ganglion and cochlear nucleus. Hearing Res 128:190–196

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

Xiang Z, Jiang L, Kang Z (2001) Transient expression of somatostatin mRNA in developing ganglion cell layers of rat retina. Dev Brain Res 128:25–33

Zhong Y, Dunn PM, Xiang Z, Bo X, Burnstock G (1998) Pharmacological and molecular characterization of P2X

purinoceptors in rat pelvic ganglion neurons. Br J Pharmacol 125:771–781

Zhong Y, Dunn PM, Burnstock G (2000a) Sympathetic neurons express varying proportions of two distinct P2X receptors. J Physiol 523:391–402

Zhong Y, Dunn PM, Burnstock G (2000b) Pharmacological comparison of P2X receptors on rat celiac, mouse celiac and mouse pelvic ganglion neurons. Neuropharmacology 39:172–180

Zhong Y, Banning AS, Cockayne DA, Ford APDW, Burnstock G, McMahon SB (2003) Bladder and cutaneous sensory neurons of the rat express different functional P2X receptors. Neuroscience (in press)

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

Zhou X, Galligan JJ (1998) Non-additive interaction between nicotinic cholinergic and P2X purine receptors in enteric neurons in culture. J Physiol 513:685–697