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The distribution of purine $P2X_2$ receptors in the guinea-pig enteric nervous system

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Abstract The $P2X_2$ subtype of purine receptor was localised by immunohistochemistry to nerve cells of the myenteric ganglia of the stomach, small and large intestines of the guinea-pig, and nerve cells of submucosal ganglia in the intestine. Nerve cells with strong and with weak immunoreactivity could be distinguished. Immunoreactivity in both strongly and weakly immunoreactive neurons was absorbed with $P2X_2$ receptor peptide. In the myenteric plexus, strong immunoreactivity was in nitric oxide synthase (NOS)- and in calbindin-immunoreactive neurons. In all regions, over 90% of NOS-immunoreactive neurons were strongly $P2X_2$ receptor immunoreactive. The intensity of reaction varied in calbindin neurons; in the ileum, 90% were immunoreactive for the receptor, about one-third having a strong reaction. In the submucosal ganglia, all vasoactive intestinal peptide-immunoreactive neurons were $P2X_2$ receptor immunoreactive, but there was no receptor immunoreactivity of calretinin or neuropeptide Y neurons. Varicose nerve fibres with $P2X_2$ receptor immunoreactivity were found in the gastric myenteric ganglia. These fibres disappeared after vagus nerve section. It is concluded that the $P2X_2$ receptor is expressed by specific subtypes of enteric neurons, including inhibitory motor neurons, non-cholinergic secretomotor neurons and intrinsic primary afferent neurons, and that the receptor also occurs on the endings of vagal afferent fibres in the stomach.

Keywords Enteric nervous system · Purine receptors · ATP · Nitric oxide synthase · Calbindin

Introduction

Two classes of receptors mediate extracellular effects of adenosine triphosphate (ATP): P2X (ionotropic) and P2Y (metabotropic) receptors. There are seven known P2X receptor subunits ($P2X_{1–7}$), which can form both homomeric and heteromeric channels, with different pharmacological properties, including different responses to agonists and antagonists, and differences in desensitisation properties (Ralevic and Burnstock 1998). The effects of agonists, and the rapid nature of excitatory synaptic events that have been attributed to ATP in enteric neurons, suggest that the dominant receptor types are of the P2X class (see below).

Although pharmacological data indicate that ATP acts on many enteric neurons, which receptors mediate these effects and which neurons bear the receptors is unclear. The enteric nervous system contains intrinsic sensory neurons, interneurons and motor neurons and, in the guinea-pig small intestine where these have been most thoroughly studied, there are 14 functionally defined neuron types that form the intrinsic circuits that control motility, transmucosal fluid transport and local blood flow (Furness 2000). On electrophysiological criteria, the neurons can be separated into two groups: AH neurons (the intrinsic sensory neurons) and S neurons (interneurons and motor neurons). AH stands for after-hyperpolarising, and is used because the AH neurons have prominent after-hyperpolarisations following action potentials. S is for synaptic, and is used to designate neurons with prominent fast synaptic inputs. Intrinsic sensory neurons are also referred to as intrinsic primary afferent neurons (IPANs).

ATP depolarises myenteric S neurons in the guinea-pig small intestine (Katayama and Morita 1989; Barajas-López et al. 1996), but the responses of AH neurons depend on the method of application. In experiments conducted at 35–37°C, bath-applied ATP hyperpolarised AH neurons (Katayama and Morita 1989). This is probably because ATP caused an opening of Ca^{2+} channels and the Ca^{2+} in turn activated a Ca^{2+}-sensitive K^+ current (Christofi et al. 1997). On the other hand, brief, focal applications of ATP, in experiments conducted at 23°C, depolarised AH neurons (Barajas-López et al. 1996). ATP also enhanced the post-action potential hyperpolarising

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current in myenteric AH neurons (Katayama and Morita 1989). This is possibly due to the entry of Ca^{2+}. Overall, ATP affects the membrane potential of 80–90% of myenteric neurons (Katayama and Morita 1989; Barajas-López et al. 1996). The majority of neurons, both AH and S, are depolarised in the submucosal ganglia (Barajas-López et al. 1994; Glushakov et al. 1998). A slower depolarisation was observed in some submucosal S neurons, which is probably mediated through P2Y receptors.

The fast depolarising effects of ATP appear to be through opening of non-specific cation channels in both myenteric (Zhou and Galligan 1996) and submucosal neurons (Barajas-López et al. 1994), which would be consistent with an action on P2X receptors. Furthermore, comparisons of agonist potencies and susceptibility to antagonists confirm that effects on both myenteric and submucosal neurons are mediated through P2X receptors (Barajas-López et al. 1996, 2000). However, both in myenteric and submucosal neurons, the 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). S neurons include several classes of interneurons and motor neurons, but which of these neurons are affected by P2X receptor agonists has never been determined.

Pharmacological analysis of transmission to enteric neurons has revealed that several transmitters contribute to fast excitatory post-synaptic potentials (fast EPSPs; Galligan and Bertrand 1994; Lepard et al. 1997; Liu et al. 1997; Lepard and Galligan 1999). In some neurons, the fast EPSPs are blocked by hexamethonium, and are thus considered to be cholinergic. However, in other neurons of the guinea-pig small intestine, fast EPSPs that have components blocked by purine receptor blockers (suramin and PPADS) have been reported (Lepard et al. 1997; Lepard and Galligan 1999). The purinergic fast EPSPs appear to be confined to descending neurons in the guinea-pig small intestine (Johnson et al. 1999). In that study, neurons were recorded from and then filled with a marker dye. Hexamethonium abolished fast EPSPs in orally projecting neurons, whereas fast EPSPs in anally projecting neurons were almost unaffected. The fast EPSPs in anally projecting neurons were almost abolished by the ATP receptor blocker, suramin (100 μM).

In the present work, we have used an antiserum specific to the $P2X_2$ subtype of P2X receptor, to determine whether the pharmacological effects that have been reported might involve this receptor subtype, and to determine which neurons bear the receptor.

Materials and methods

Tissue was obtained from 24 guinea-pigs of both genders, in the weight range 200–400 g, that were stunned by a blow to the head and killed by severing the carotid arteries and spinal cord. All procedures were approved by the University of Melbourne Animal Experimentation Ethics Committee. Segments of stomach, duodenum, distal ileum, caecum, proximal colon, distal colon and rectum were removed and placed in phosphate-buffered saline (PBS; 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2) to which was added nicardipine (10^{-6} M; Sigma, Castle Hill, Sydney, Australia) in order to prevent tissue contraction. Tissue was then opened and cleaned of contents, pinned tautly on balsa board, mucosa side down, and immersed in 2% formaldehyde plus 0.2% picric acid in 0.1 M sodium phosphate buffer, pH 7.0, at 4°C overnight. Some samples of ileum were also fixed without stretching, so that cryostat sections could be taken. The next day, tissue was cleared of fixative with 3×10 min washes in dimethylsulphoxide, followed by 3×10 min washes in PBS. Tissue was stored in PBS containing sodium azide (0.1%) at 4°C.

Subdiaphragmatic truncal vagotomy

Bilateral vagal trunk section was performed on two guinea-pigs following the procedure previously described (Furness et al. 2001). Food was withdrawn on the day prior to surgery, and was restored on the day after. Prior to surgery, guinea-pigs were anaesthetised with a mixture of xylazine (10 mg/kg) and ketamine (50 mg/kg), given intramuscularly. The peritoneal cavity was opened in the abdominal midline, and the left and right vagal trunks were located anterior and posterior to the oesophagus, below the diaphragm, and a length of about 1 cm of nerve was excised. In addition, connective tissue was removed from the surface of the oesophagus, around its circumference. A pyloromyotomy was also performed, by slitting the muscle of the pyloric sphincter with a cut to the depth of the submucosa, extending from the antrum to the duodenum. This is to allow gastric emptying to occur after the vagotomy. The animals were killed and tissue was taken 9 days after the operation.

Fluorescence immunohistochemistry

Following fixation and clearing, the tissue was dissected into layers. The mucosa, submucosa and circular muscle were removed to produce whole mounts of longitudinal muscle plus the myenteric plexus. In the second type of preparation, the mucosa and muscularis externa were removed to leave behind the intact submucosa. To prepare the tissue for sectioning, small segments of ileum were transferred to a mixture of PBS-sucrose-azide and OCT compound (Tissue Tek, Elkhart, Ind., USA) in a ratio of 1:1 for 24 h before being embedded in 100% OCT. Sections of 12 μm thickness were cut and collected on slides coated with aminopropyltriethoxysilane (Sigma Chemicals, St. Louis, Mo., USA) and left to dry for 1 h at room temperature. All preparations were incubated in a 10% solution of normal horse serum plus 1% Triton X-100 in PBS for 30 min at room temperature, prior to exposure to primary antisera (Table 1).

To localise $P2X_2$ receptor immunoreactivity, we used a rabbit antiserum raised against amino acid sequence 457–472 of the rat $P2X_2$ receptor, with a single Cys extension at the N-terminal (AB5244 from Chemicon, Temecula, Calif., USA). Incubation was for 48 h at 4°C at a dilution of 1:120.

Double labelling was achieved using combinations of antisera (Table 1). Following incubation in primary antisera, tissue was given 3×10 min washes in PBS and then incubated in a mixture of secondary antibodies (see Table 2). Further 3×10 min washes in PBS were made before tissue was mounted in glycerol buffered with 0.5 M sodium carbonate buffer (pH 8.6).

To test the specificity of the immunohistochemical reaction, antiserum diluted to the concentration used for immunohistochemistry was combined with the synthetic peptide, N-cys-457–472, of the rat $P2X_2$ receptor, at a concentration of 4×10^{-5} M and equilibrated at 4°C overnight. Primary antiserum with no absorbing peptide was used in parallel experiments as a control.

Preparations were examined on a Zeiss Axioplan microscope equipped with the appropriate filter cubes for discriminating be-

Table 1 Characteristics of primary antibodies. (*NOS* nitric oxide synthase, *NPY* neuropeptide Y, *VIP* vasoactive intestinal peptide)

Tissue antigen	Host	Dilution	Code and reference
Calbindin	Mouse	1:500	Swant 300, batch 17-F against chicken intestinal calbindin 28 k (Reiche et al. 1999)
Calbindin	Sheep	1:800	PES1 (Furness et al. 1989)
Calretinin	Goat	1:100	CG1, Swant
NOS	Sheep	1:2,000	H205 (Williamson et al. 1996)
NPY	Sheep	1:400	E2210 (Furness et al. 1985)
$P2X_2$	Rabbit	1:120	AB5244, Chemicon
VIP31	Mouse	1:1,000	Gift from Dr. Alison Buchan

Table 2 Secondary antibodies used. Supply companies: Amersham Pty, Melbourne, Australia; Molecular Probes, Eugene, Ore., USA. (*FITC* Fluorescein isothiocyanate)

Antibody	Dilution	Source
Donkey anti-rabbit IgG FITC	1:50	Amersham
Donkey anti-sheep IgG Alexa 594	1:50	Molecular Probes
Goat anti-mouse IgG Alexa 594	1:200	Molecular Probes

tween fluorescein isothiocyanate (FITC) and Alexa 594 fluorescence. We used filter set 10 for FITC (450–490 nm excitation filter and 515–565 nm emission filter), and filter set 00 for Alexa 594 (530–585 nm excitation filter and 615 nm emission filter). Images were recorded using a SpotRT cooled charge-coupled device camera and SpotRT 3.2 software (Diagnostic Instruments, Sterling Heights, Mich., USA). Preparations were also analysed by confocal microscopy on a Biorad MRC1024 confocal scanning laser system installed on a Zeiss Axioplan 2 microscope. The system had a krypton/argon laser for differential visualisation of the fluorophores using 488 nm excitation filter and 522/535 nm emission filter for FITC and 568 nm excitation filter and 605/632 nm emission filter for Alexa 594. The images were 512×512 pixels and the thickness of each optical section was 0.5 µm. Immunoreactive cells were scanned as a series of optical sections with a centre to centre spacing of 0.2 µm. Confocal images were collected using Biorad Lasersharp processing software. Images were further processed using Confocal Assistant, Corel PhotoPaint and Corel Draw software programs.

Quantitative analysis

The proportions of neurons in which antigen immunoreactivity was colocalised were determined by examining fluorescently labelled, double-stained preparations. Neurons were first located by the presence of a fluorophore that labelled one antigen, and then the filter was switched to determine whether or not the neuron was labelled for a second antigen, located with a second fluorophore of a different colour. In this way, proportions of neurons labelled for pairs of antigens were determined. Cohort size was 50 neurons and data was collected from preparations obtained from at least three animals. The percentage of neurons immunoreactive for a particular marker that was also immunoreactive for a second neurochemical was calculated and expressed as mean ± standard error of the mean (SEM; *n*=number of preparations counted).

Results

Immunoreactivity occurred in nerve cell bodies in all regions that were examined, namely the stomach, duodenum, ileum, caecum, proximal colon, distal colon and rectum myenteric plexus, and in the ileum and distal colon submucosal plexus. In the small and large intestines, the varicose nerve fibres that surround nerve cells were not immunoreactive, although some non-varicose processes of cell bodies were immunoreactive, and a low level of immunoreactivity was observed in nerve bundles passing through the ganglia. Reactive varicose fibres surrounding nerve cells were only observed in the myenteric plexus of the stomach.

The immunoreactive nerve cells could be easily separated into strongly and weakly immunoreactive subgroups (Fig. 1). The difference between the two groups was measured using a pixel intensity function of the Biorad Lasersharp processing software. For ten strongly and ten weakly immunoreactive nerve cells in the myenteric plexus of the ileum, pixel intensities were 146±13 (mean ± SD), range 126.3–154.1, for strongly immunoreactive cells, and 98±10, range 85.9–114.0, for weakly immunoreactive cells. These data are significantly different (*t*-test, $P<0.001$). This confirmed the visual impression that the neurons form two groups, based on their fluorescence intensity. Immunoreactivity occurred throughout the cytoplasm and on the surface membranes of the nerve cells. Immunoreactivity of both the strongly and weakly reactive nerve cells was lost if the antiserum was pre-equilibrated with the peptide against which the antiserum was raised.

Sections were taken through the wall of the small intestine to determine whether $P2X_2$ immunoreactivity occurred on epithelial cells, particularly entero-endocrine cells. We were unable to detect immunoreactivity in the epithelium.

Double-labelling studies

Double-labelling studies were conducted to identify major classes of neurons which have strong $P2X_2$ receptor immunoreactivity in the myenteric and submucosal ganglia of the ileum and distal colon, and the myenteric plexus of the duodenum and gastric corpus (Fig. 2; Table 3).

In the ileum myenteric plexus, 75±10% (*n*=7) of strongly immunoreactive neurons were immunoreactive for nitric oxide synthase (NOS). In other regions, the majority of strongly immunoreactive myenteric neurons were also NOS immunoreactive: distal colon, 92±6% (*n*=8); duodenum, 64±13% (*n*=6); gastric corpus, 92%

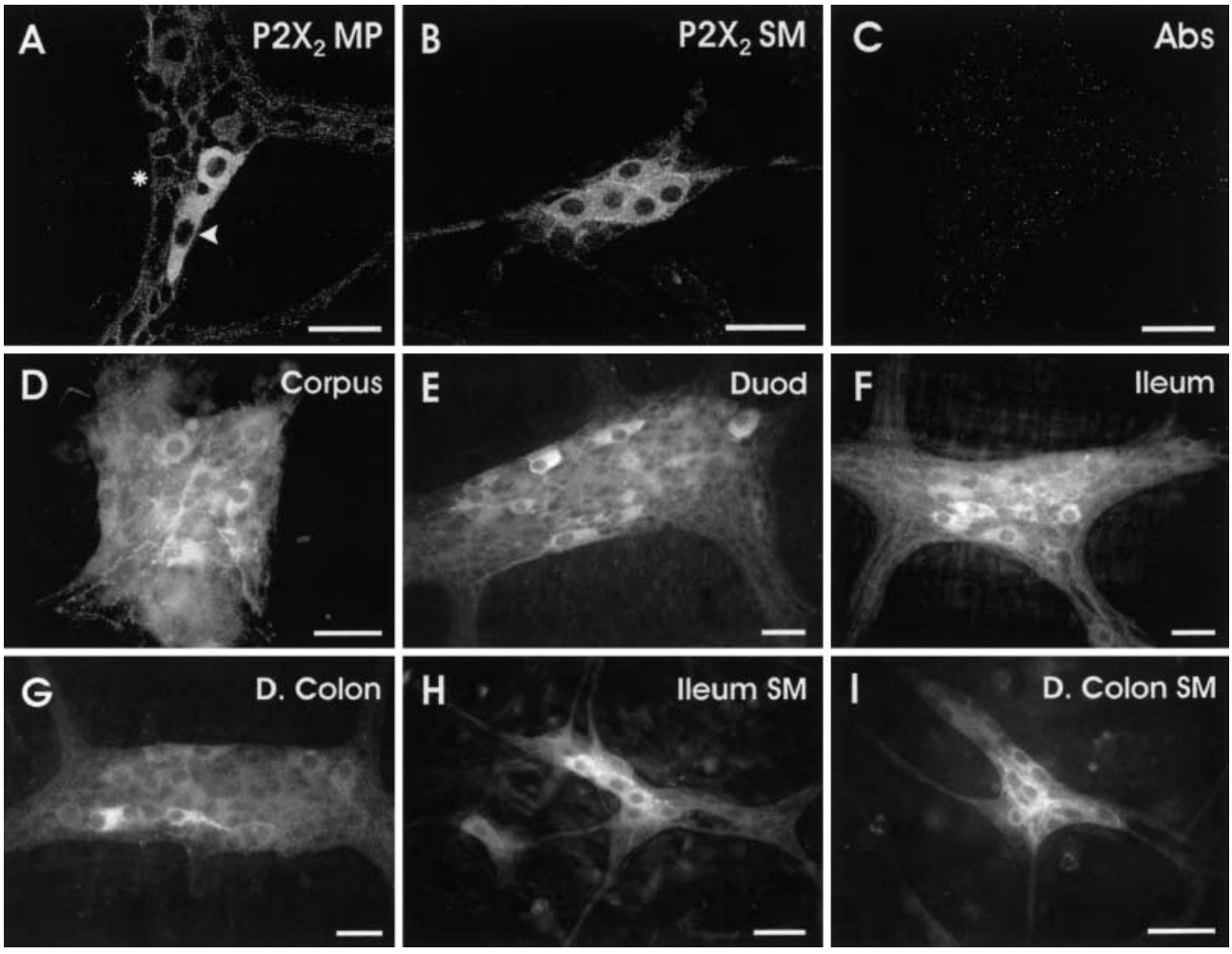

Fig. 1A–I $P2X_2$ receptor immunoreactivity in myenteric (*MP*) and submucosal (*SM*) ganglia of the guinea-pig stomach and intestine. These micrographs show that there are both strongly immunoreactive and weakly immunoreactive nerve cells in the ganglia. **A–C** Confocal images from myenteric ganglia in the ileum. Both strongly immunoreactive neurons (*arrowhead*, **A**) and weakly immunoreactive neurons (*asterisk*, **A**) were not revealed when the antiserum had been pre-equilibrated (absorbed, *Abs*) with the peptide fragment of the $P2X_2$ receptor against which the antiserum had been raised (**C**). **D–G** Conventional fluorescence images of myenteric ganglia in different regions of the gastrointestinal tract, gastric corpus (**D**), duodenum (**E**), ileum (**F**) and distal colon (**G**). In each region there are both strongly and weakly immunoreactive nerve cell bodies. **H**, **I** Immunoreactivity in cell bodies of the submucosal ganglia in the ileum (**H**) and distal colon (**I**). *Bars* 25 µm in **A–C**; 50 µm in **D–I**

Table 3 Classification of neurons with $P2X_2$ immunoreactivity, by their immunoreactivity (IR) for markers of functionally identified neurons, in ganglia of the myenteric plexus (MP) and submucosal plexus (SMP). Data expressed as mean ± SEM, with the number of cohorts of 50 neurons counted *in parentheses*

Region	Neuron type	Percent immunoreactive for $P2X_2$
Ileum MP	NOS-IR, inhibitory motor neurons and descending interneurons	91±7% (*n*=7)
	Calbindin-IR, intrinsic primary afferent neurons	90±5% (*n*=8)
Ileum SMP	NPY-IR, cholinergic secretomotor neurons	0% (*n*=3)
	Calretinin-IR, cholinergic secretomotor and vasomotor neurons	0% (*n*=3)
	VIP-IR, non-cholinergic secretomotor neurons	100% (*n*=6)
	Submucosal intrinsic primary afferent neurons	100% (*n*=6)
Distal colon MP	NOS-IR, inhibitory motor neurons and descending interneurons	96±4% (*n*=7)
	Calbindin-IR, intrinsic primary afferent neurons	91±9% (*n*=8)
Distal colon SMP	VIP-IR, non-cholinergic secretomotor neurons	100% (*n*=2)

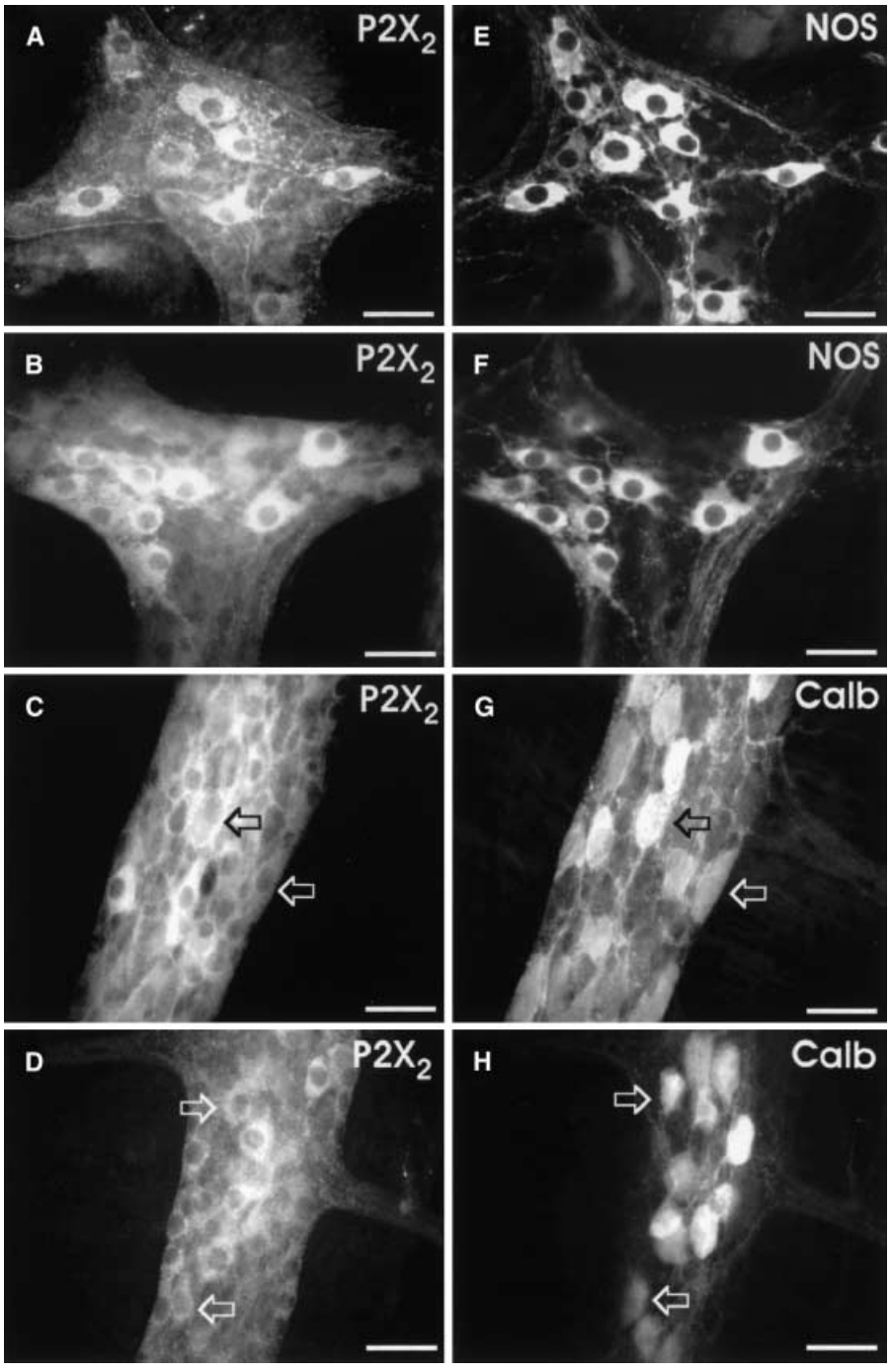

Fig. 2A–H Colocalisation of $P2X_2$ receptor immunoreactivity with nitric oxide synthase (*NOS*) immunoreactivity and calbindin (*Calb*) immunoreactivity in myenteric ganglia. **A**, **E** Colocalisation with NOS in the gastric corpus. **B**, **F** Colocalisation with NOS in neurons of the duodenum. The majority of NOS-immunoreactive neurons had strong $P2X_2$ receptor immunoreactivity. **C**, **G** Colocalisation with calbindin in the ileum. **D**, **H** Colocalisation with calbindin in the distal colon. Double-labelled neurons are indicated by the *arrows*. *Bars* 50 µm

and 100% (n=2). Conversely, the majority of NOS-immunoreactive neurons were immunoreactive for the $P2X_2$ receptor: ileum, 91±7% (n=7); distal colon, 96±4% (n=7); duodenum, 98±2% (n=6); gastric corpus, 88% and 94% (n=2).

Of the remaining neurons with strong $P2X_2$ immunoreactivity, most were calbindin immunoreactive (Fig. 2). However, there were also calbindin-immunoreactive neurons that had weak immunoreactivity for the receptor. Of calbindin neurons in the myenteric plexus of the ileum, 90±5% (n=8) were $P2X_2$ receptor immunoreactive, about one-third of these being strongly immunoreactive and two-thirds weakly reactive. The proportions of calbindin-immunoreactive neurons with $P2X_2$ receptor immunoreactivity in the myenteric plexus of the distal colon (91±9%; n=8) and duodenum (91±7%; n=7) were similar to that in the ileum. Calbindin-immunoreactive neurons are rare in the stomach, and colocalisation with $P2X_2$ receptor immunoreactivity was not tested.

$P2X_2$ receptor-immunoreactive neurons in submucosal ganglia of the ileum were all strongly immunoreactive. Double staining showed that none of these were

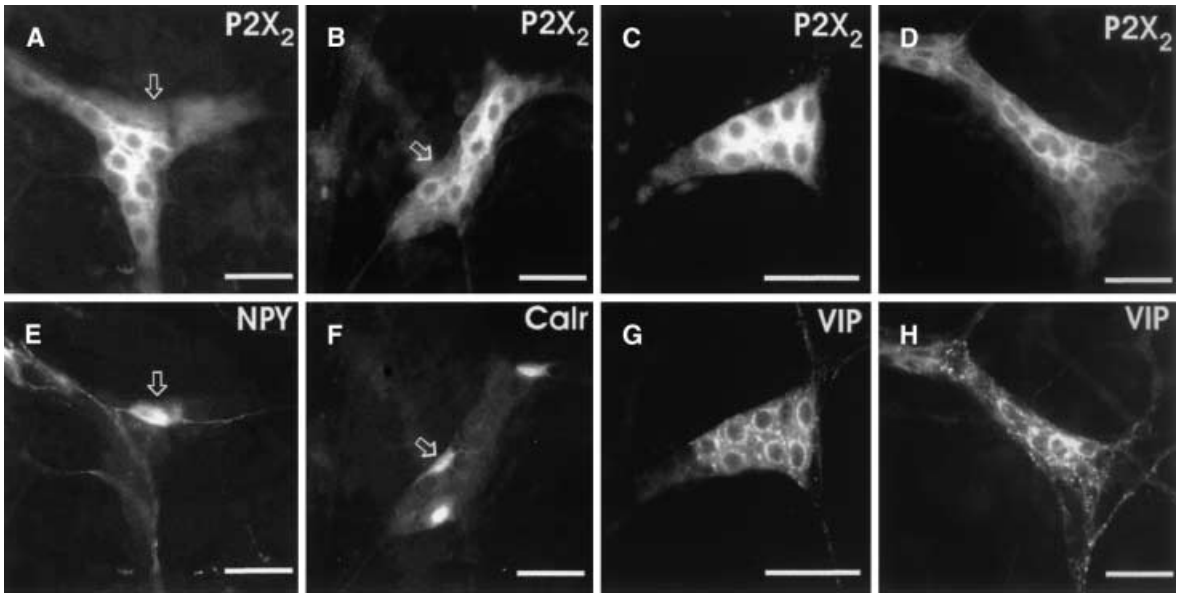

Fig. 3A–H Colocalisation of $P2X_2$ receptor immunoreactivity in nerve cells of submucosal ganglia. In submucosal ganglia of the ileum, nerve cells with strong immunoreactivity for the $P2X_2$ receptor were not immunoreactive for neuropeptide Y (*NPY*; **A**, **E**) or for calretinin (**B**, **F**). *Arrows* indicate neurons only reactive for NPY or calretinin. However, all vasoactive intestinal peptide (*VIP*)-immunoreactive neurons had $P2X_2$ receptor immunoreactivity in the ileum (**C**, **G**) and distal colon (**D**, **H**). *Bars* 50 µm

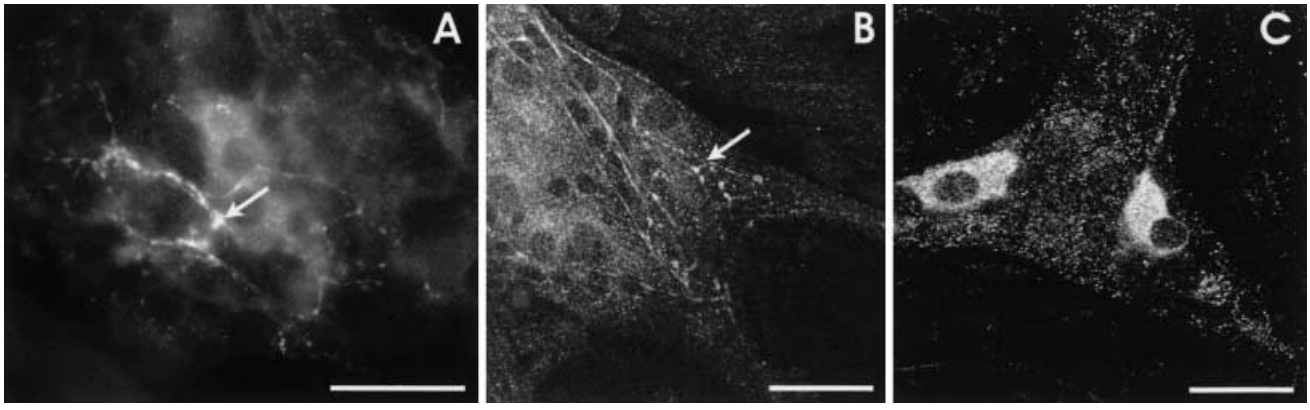

Fig. 4A–C Varicose fibres with $P2X_2$ receptor immunoreactivity in the gastric myenteric plexus. **A** Conventional fluorescence image, showing varicosities around a nerve cell. Some varicosities were large (*arrow*). **B** Confocal image of $P2X_2$ receptor-immunoreactive varicosities in a myenteric ganglion. **C** Confocal image of $P2X_2$ receptor immunoreactivity in a ganglion from the stomach after subdiaphragmatic section of the vagus nerve. The immunoreactive varicosities disappeared following vagotomy, but reactive nerve cells remained. *Bars* 50 µm

neuropeptide Y (NPY) immunoreactive, and none were calretinin immunoreactive (Fig. 3). On the other hand, all vasoactive intestinal peptide (VIP)-immunoreactive nerve cells in the submucosal ganglia were $P2X_2$ receptor immunoreactive (100% in each one of six preparations). However, 10±4% (n=6) of $P2X_2$-immunoreactive submucosal nerve cells were not immunoreactive for VIP. These cells were large and oval in profile. We also examined the submucosal plexus of the distal colon. Here, there were also strongly $P2X_2$ receptor immunoreactive neurons, and, as in the ileum, all VIP-immunoreactive nerve cells had $P2X_2$ receptor immunoreactivity (cells counted in two preparations). There were a small number of $P2X_2$ receptor-immunoreactive nerve cells that were not immunoreactive for VIP.

Immunoreactive fibres in the stomach

Immunoreactive nerve fibres were observed in the gastric myenteric plexus (Fig. 4). These fibres surrounded some myenteric nerve cells. Because some of the varicosities were large and irregular, similar to the endings of vagal afferent fibres (Berthoud et al. 1997), we performed vagotomies in two guinea-pigs to determine whether the fibres were of vagal origin. The fibres were no longer present 9 days after vagal nerve section, although in the same tissue the immunoreactive nerve cell bodies were still apparent (Fig. 4). These data are consis-

tent with the observation that $P2X_2$ receptor mRNA and $P2X_2$ receptor immunoreactivity occur in the vagal nodose ganglion (Nörenberg and Illes 2000).

Discussion

The present work shows that a substantial proportion of enteric neurons contains $P2X_2$ receptor immunoreactivity. In the ileum, where electrophysiological studies have been made of the effects of P2X receptor agonists, the distribution can be compared with that of functional receptors. In the myenteric plexus, electrophysiological examination has shown P2X receptors to occur in about 80–90% of neurons (Katayama and Morita 1989; Barajas-López et al. 1996). Immunoreactivity for the $P2X_2$ isoform occurred in a smaller population, and about 30% of the neurons were strongly $P2X_2$ immunoreactive. There is a similar discrepancy in submucosal ganglia, where 55% of the neurons were immunoreactive for the $P2X_2$ receptor subtype, although about 90% of the neurons have receptors of the P2X group, judging from electrophysiological data (Barajas-López et al. 1994; Glushakov et al. 1998). The results suggest that the types of P2X receptors are specific to particular subgroups of neurons. In the myenteric plexus, the $P2X_2$ subtype is in inhibitory muscle motor neurons and intrinsic sensory neurons (see discussion below). Together these make up about 40% of myenteric neurons (Furness 2000). The remaining myenteric neurons that have P2X receptors (a further 40–50%, including longitudinal muscle motor neurons and excitatory neurons innervating the circular muscle), presumably exhibit another isoform. Some of these may include neurons with weak $P2X_2$ immunoreactivity. It is possible that the weakly immunoreactive neurons have heteromeric receptors and that the strongly immunoreactive neurons perhaps express homomeric $P2X_2$ receptors (Ralevic and Burnstock 1998). The submucosal neurons are more clearly distinguishable. The VIP-containing secretomotor neurons and the calbindin–tachykinin neurons were $P2X_2$ receptor immunoreactive (see below). The observation that the types of $P2X_2$ receptor isomers differ in neurons with different physiological roles implies that it might be possible to develop drugs to target one neuron type. It is already established that the subtypes of P2X receptors have different pharmacological profiles (Ralevic and Burnstock 1998).

The types of neuron that bear the $P2X_2$ receptor type can be deduced from previous studies of the relation between neurochemistry and function of enteric neurons of the guinea-pig ileum (Furness 2000). In the myenteric ganglia of the ileum, 90% of NOS neurons were strongly $P2X_2$ positive, and the corresponding figure in the duodenum was 98%. These data indicate that $P2X_2$ receptor occurs on inhibitory motor neurons that innervate the circular muscle. In the duodenum, all NOS-immunoreactive neurons with cell bodies in myenteric ganglia are inhibitory motor neurons supplying the circular muscle (Clerc et al. 1998), and in the ileum, 75% of the NOS neurons are inhibitory motor neurons, the remainder being descending interneurons (Li and Furness 2000). The identification of $P2X_2$ receptors on NOS neurons is consistent with studies that demonstrate suramin-sensitive fast EPSPs in descending neurons (Johnson et al. 1999). Most of these neurons (seven of eight) had Dogiel type I morphology, which is the morphology of all NOS neurons (Furness et al. 1994).

$P2X_2$ receptor immunoreactivity occurred on calbindin-immunoreactive myenteric neurons. The calbindin neurons are Dogiel type II neurons that have AH electrophysiological characteristics (Iyer et al. 1988). This result is consistent with functional evidence for the presence of P2X-type receptors on AH neurons (Barajas-López et al. 1996).

Four classes of nerve cell bodies account for almost all neurons in the submucosal ganglia of the guinea-pig ileum (Furness et al. 1984; Quinson et al. 2001). These are: (1) Dogiel type II neurons, immunoreactive for choline acetyltransferase (ChAT), tachykinins and calbindin (about 10% of nerve cells), (2) neurons immunoreactive for calretinin and ChAT (about 15%), (3) neurons with VIP immunoreactivity (about 45%) and (4) neurons with both NPY and ChAT immunoreactivity (about 30%). $P2X_2$ receptor immunoreactivity was absent from both calretinin and NPY-immunoreactive neurons. By contrast, all VIP-immunoreactive neurons were immunoreactive for the receptor (which was also the case in the colon submucosal ganglia). However, about 12% of receptor-immunoreactive neurons in the ileum were not VIP immunoreactive. As no cells were calretinin or NPY positive, this 12% of $P2X_2$ receptor immunoreactive cells must be the Dogiel type II neurons. The 12% of $P2X_2$-immunoreactive neurons which is not VIP, calretinin or NPY immunoreactive is 5% of all neurons. Thus about half of the 10% of neurons that are Dogiel type II bear the $P2X_2$ receptor.

In conclusion, strong expression of $P2X_2$ receptors was confined to about 25% of myenteric and 50% of submucosal neurons in the ileum. This contrasts with electrophysiological and calcium imaging studies that indicate that 90% of the neurons have P2X-type purine receptors. The results thus imply that other isoforms of P2X receptors are on some of the neurons, and that the type of P2X receptor is specific to functional subtypes of enteric neuron, the $P2X_2$-type being on inhibitory motor neurons and IPANs in the myenteric ganglia and noncholinergic secretomotor neurons and IPANs in the submucosa.

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