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$P2X_2$ and $P2X_3$ receptor expression in the gallbladder of the guinea pig

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Abstract

We investigated for the first time, the distribution pattern of $P2X_2$ and $P2X_3$ receptors in the gallbladder of the guinea pig using immunohistochemistry. $P2X_2$ and $P2X_3$ receptor-immunoreactive nerve fibers were observed within the ganglia, in the interganglionic connectives, in the muscularis and in the paravascular plexus. Immunoreactivity for $P2X_2$ and $P2X_3$ was also observed in most neurons in the ganglionated plexus. Double-labeling studies revealed that 58.1% of all $P2X_2$-positive neurons and 54.3% of all $P2X_3$-positive neurons were found to display nitric oxide synthase. Over 90% of the neurons that were immunoreactive for $P2X_2$ and $P2X_3$ receptor were also immunoreactive for calretinin. We also found that 30.5% of $P2X_2$- and 32.6% of $P2X_3$-immunoreactive neurons were also immunoreactive for vasoactive intestinal peptide. No $P2X_2$- or $P2X_3$- immunoreactive neurons stained for calcitonin gene-related peptide; a few calcitonin gene-related peptide-immunoreactive nerve fibers also showed immunoreactivity to $P2X_2$ or $P2X_3$ receptors. These results further demonstrate the neurotransmitter diversity of the nerves of the gallbladder and provide an incentive for studies of the actions of these compounds in the gallbladder wall.
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1. Introduction

Adenosine 5′-triphosphate (ATP) acts as an extracellular signalling molecule in both neuronal and non-neuronal systems (Abbracchio and Burnstock, 1998; Ralevic and Burnstock, 1998). 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 ($P2X_1$–$P2X_7$), which can assemble to form homomeric and heteromeric receptors, have been identified by molecular cloning (Khakh et al., 2001; Burnstock, 2003a). The subunit composition of P2X receptors determines their pharmacological and functional properties (North, 2002). The $P2X_3$ subunit can form functional homomeric complex channels when expressed on its own or heteromeric complexes with $P2X_2$ (Lewis et al., 1995). For example, the stable ATP analogue α, β-methylene ATP causes rapidly desensitizing inward currents in cells expressing homomeric $P2X_3$ receptors, but does not activate homomeric $P2X_2$ receptors (North and Surprenant, 2000). When $P2X_3$ and $P2X_2$ subunits are co-expressed as a heteromeric receptor, α, β-methylene ATP causes a biphasic response consisting of rapidly desensitizing and slowly desensitizing components (Lewis et al., 1995; North and Surprenant, 2000). Immunohistochemical studies in rodents have shown $P2X_2$ and $P2X_3$-like immunoreactivity in a widespread distribution. In guinea pig, $P2X_2$ and $P2X_3$ receptors have been detected in ganglia of the enteric nervous system (Castelucci et al., 2002; Van Nassauw et al., 2002). However, no studies of the subunit composition of P2X receptors expressed have been conducted in gallbladder.

Recent evidence suggests that the ganglia, derived from the enteric nervous system, in the wall of the gallbladder are important targets for regulatory signals that influence motor behaviour (Mawe et al., 1994, 1997). The ganglionated plexus of the guinea pig gallbladder consists of a network of small, irregularly shaped ganglia that are situated at the outer surface of the smooth muscle layer (Cai and Gabella, 1983, 1984; Mawe and Gershon, 1989). These ganglia have an appearance similar to submucosal ganglia of the intestine, but the resident neurons that number between 2 and 10 per ganglion, are larger, measuring 25–35 μm in diameter. Gallbladder ganglia are interconnected by tracts of unmy-

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elinated axons, which are contiguous with paravascular nerve bundles that follow the extensive vascular distribution in this layer. A neural plexus of the gallbladder muscularis is sparse in the guinea pig. A rich network of nerve fibers lies in the lamina propria, with branching nerve fibers in close apposition with epithelial cells.

The ganglia of the guinea pig gallbladder are comprised of two sets of neurons, based on selective expression of immunoreactivity for certain neuroactive compounds (Talmage and Mawe, 1993; Talmage et al., 1992, 1996; Mawe et al., 1997). One set of neurons, representing the majority of the total population, expresses substance P-, neuropeptide Y- and somatostatin-like immunoreactivity, and the other set of neurons expresses vasoactive intestinal peptide (VIP)-like immunoreactivity and nitric oxide synthase- (NOS) immunoreactivity and stains positively for NADPH-diaphorase. The former set of neurons may represent a group of excitatory ganglion cells with the latter set of neurons having an inhibitory role (Talmage and Mawe, 1993; Talmage et al., 1996).

In this study, we have stained whole mount preparations for $P2X_2$ and $P2X_3$ receptors on the ganglionated plexuses in the gallbladders of guinea pigs. In addition, we have utilized double-label immunohistochemistry to examine the relationship between $P2X_2$, $P2X_3$ receptors and NOS, calcitonin gene-related peptide (CGRP), VIP and calretinin.

2. Materials and methods

2.1. Tissue preparation

Ten adult guinea pigs (200–350 g) of either sex were killed by asphyxiation with a slowly rising concentration of CO_2, and death was confirmed by cervical dislocation according to Home Office (UK) regulations covering Schedule 1. The gallbladder was quickly removed, washed with chilled Krebs' solution (pH, 7.4, in mM: NaCl, 121; KCl, 5.9; $CaCl_2$, 2.5; $MgCl_2$, 1.2; $NaHCO_3$, 25; NaH_2PO_4, 1.2; and glucose, 8), opened, and pinned flat (mucosal side up) in dishes lined with a silicone elastomer (Sylgard, Dow Corning, Midland, MI). The mucosal and underlying connective tissue layers were carefully removed using forceps under a dissecting scope. The preparations were then fixed for 4 h at 4°C in a solution containing 4% paraformaldehyde and 0.2% picric acid in 0.1 M sodium phosphate buffer at pH 7.4. The fixed tissues were washed with 0.1 M phosphate-buffered saline and further processed.

2.2. Primary antibodies directed against the $P2X_2$ and $P2X_3$ receptors

The antibodies directed against the $P2X_2$ and $P2X_3$ receptors, provided by Roche Bioscience (Palo Alto, CA, USA), have been detailed previously (Oglesby et al., 1999). The immunogens used for the production of polyclonal $P2X_2$ and $P2X_3$ antibodies were synthetic peptides corresponding to the carboxyl terminal of the cloned rat $P2X_2$ and $P2X_3$ receptors, covalently linked to keyhole limpet hemocyanin. The peptide sequences of the $P2X_2$ and $P2X_3$ receptors are of amino acid sequence 458–472 (QQDSTSTDPKGLAQL) and 383–397 (VEKQSTDSGAYSIGH), respectively. The polyclonal antibodies were raised by multiple, monthly injections of New Zealand rabbits with the corresponding peptides (prepared by Research Genetics, Huntsville, AL). The specificity of the antisera was verified by immunoblotting with membrane preparations from CHO K1 cells expressing the cloned $P2X_2$ and $P2X_3$ receptors (Oglesby et al., 1999). As previously reported by Oglesby et al. (1999), no cross-reactivity is observed with these antisera.

2.3. Immunocytochemistry

The antisera used in this study as well as their respective dilutions, are listed in Table 1. The preparations were washed 3×5 min in PBS, then preincubated with 0.5% Triton X-100 in phosphate-buffered saline (0.1 M; PBS) for 30 min. Preparations were then incubated in the primary antibodies ($P2X_2$ or $P2X_3$) diluted to 3 μg/ml with 10% normal horse serum (NHS) in PBS containing 0.05% merthiolate and 0.2% Triton X-100 (4 °C, 24 h). Subsequently, the preparations were incubated with biotinylated donkey anti-rabbit IgG, diluted 1:500 in 1% NHS in PBS containing 0.05% merthiolate for 1 h, followed by incubation in Streptavidin-Fluorescein (FITC-green fluorophore) diluted 1:200 in PBS containing 0.05% merthiolate for 1 h.

Table 1
List of antisera used for immunocytochemistry

Antigen	Host	Dilution	Source
Primary antisera			
$P2X_2$ receptor	Rabbit	1:400	Roche Bioscience Palo Alto Calif., USA
$P2X_3$ receptor	Rabbit	1:400	Roche Bioscience Palo Alto
Neuronal nitric oxide synthase	Sheep	1:800	Santa Cruz Biotechnology, Santa Cruz, CA
Calretinin	Mouse	1:2000	Swant, Bellinzola, Switzerland
Vasoactive intestinal peptide	Sheep	1:400	Biogenesis, Sandown, USA
Calcitonin gene-related peptide	Mouse	1:2000	Affiniti, Mamhead, UK
Secondary antisera and streptavidin complexes			
Biotinylated donkey anti-rabbit IgG		1:500	Jackson ImmunoResearch Lab, West Grove, PA, USA
Cy3-conjugated goat anti-mouse IgG		1:200	Jackson ImmunoResearch Lab
Cy3-conjugated donkey anti-sheep IgG		1:200	Jackson ImmunoResearch Lab
Streptavidin-Fluorescein (FITC-green fluorophore)		1:200	Amersham Biosciences, UK

All incubations were held at room temperature and separated by three 5-min washes in PBS. Slides were mounted with Citifluor (Citifluor, London, UK) and examined with fluorescence microscopy.

To demonstrate the colocalization of the $P2X_2$ or $P2X_3$ receptor with neuronal NOS, calretinin, VIP and CGRP, preparations were immunostained for the $P2X_2$ or $P2X_3$ receptor, as above, then incubated with appropriate antibodies overnight. Subsequently, the preparations were incubated with Cy3-conjugated goat anti-mouse IgG or Cy3-conjugated donkey anti-sheep IgG. All the incubations and reactions were held at room temperature and separated by 3×10-min washes in PBS. The preparations were mounted with Citifluor and examined with fluorescence microscopy.

Control experiments included preabsorption of primary antisera with the peptide they were originally raised against.

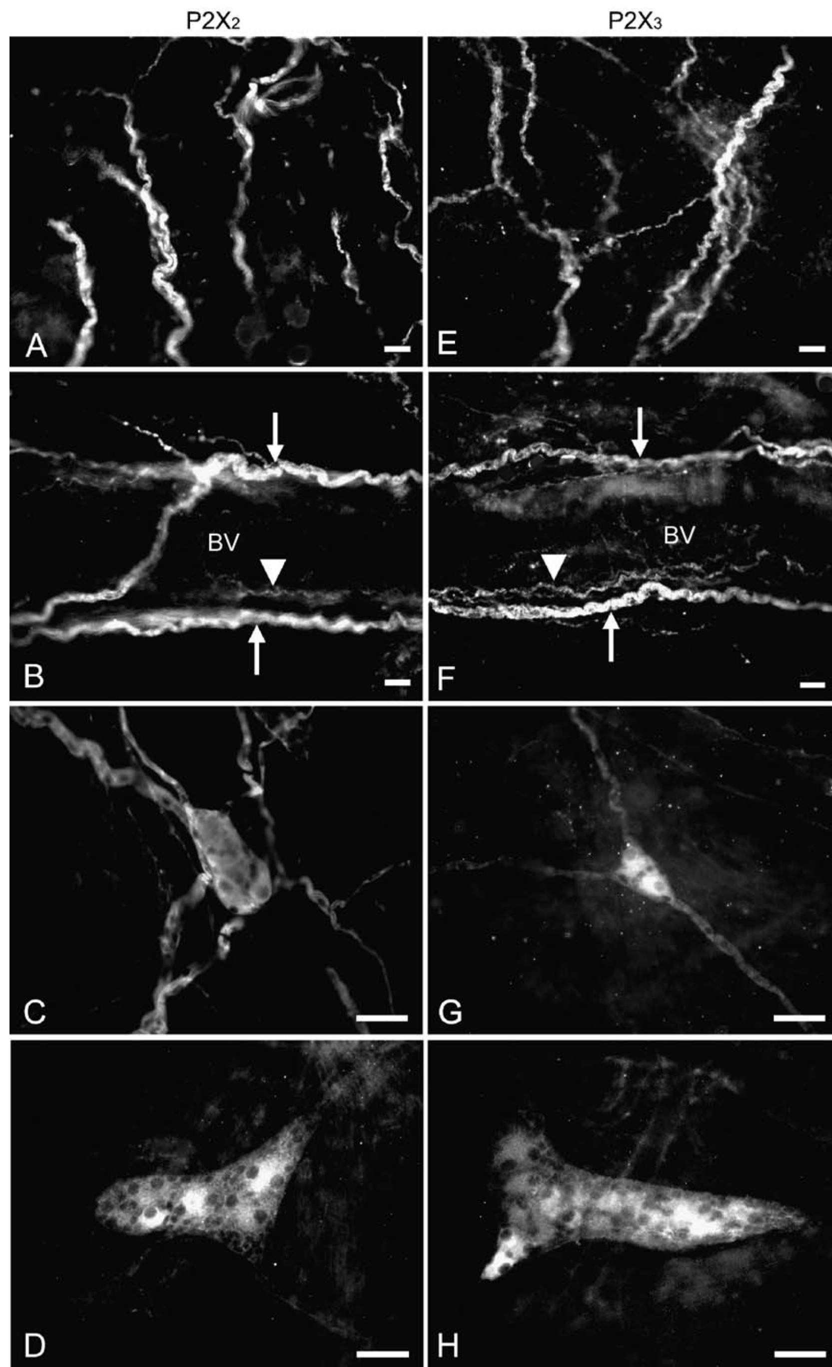

Fig. 1. Examples of $P2X_2$ and $P2X_3$ receptor immunoreactivity in the guinea pig gallbladder. (A, E) $P2X_2$ and $P2X_3$ receptor immunoreactive nerve bundles in the muscle layer. (B, F) Large blood vessels (BV) showing both a perivascular plexus (arrowheads) and large nerve bundles in the outer adventitia (arrows). (C, D) $P2X_2$ and (G, H) ($P2X_3$ receptor immunoreactive nerve fibres and neuron cell bodies in the nerve plexus. Scale bar, 50 μm.

Additional control experiments involved omission of the primary antibody.

2.4. Photomicroscopy

Images of immunofluorescence labeling were taken with the Leica DC 200 digital camera (Leica, Switzerland) attached to a Zeiss Axioplan microscope (Zeiss, Germany). Filter sets included the following: for Cy3, excitation, 510–550 nm, emission, 590 nm; for FITC, 470-nm excitation, 525-nm emission. Images were imported into a graphics package (Adobe Photoshop 5.0, USA). The two-channel readings for green and red fluorescence were merged by using Adobe-Photoshop 5.0.

2.5. Analysis

Whole mount preparations of the gallbladder nerve plexus are technically difficult to prepare. Although 20 preparations were examined, only a limited number were good enough to use for quantitative studies. However, some estimation of colocalisation was attempted. All analyses were performed at $\times 20$ 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 neuron bodies in the 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 neurons was calculated.

3. Results

We first processed whole mount preparations for $P2X_2$ and $P2X_3$ receptor immunoreactivity in guinea pig gallbladder. We observed $P2X_2$ and $P2X_3$ receptor-immunoreactive nerve fibers were abundant within the ganglia, in the interganglionic connectives and in the muscularis. The $P2X_3$-immunoreactive nerve fibers in the muscularis comprised a network of fibers. The $P2X_2$ and $P2X_3$-positive fibers could be followed along blood vessels and could be seen passing through nerve bundles linking the paravascular plexus with the ganglionated plexus (Fig. 1). Immunoreactivity for $P2X_2$ and $P2X_3$ was also observed in most neurons in the ganglionated plexus (Fig. 1). Control immunostaining, in which the primary antibodies were omitted, or were absorbed with the peptide, did not yield any immunolabelling.

Double labeling studies revealed that 58.1% of all $P2X_2$-positive neurons and 54.3% of all $P2X_3$-positive neurons were found to display NOS (Table 2; Figs. 2A–C and 3A–C).

In preparations that were co-stained for $P2X_2$ and $P2X_3$ receptor with calretinin immunoreactivity, over 90% of the neurons that were immunoreactive for $P2X_2$ and $P2X_3$ receptor were also immunoreactive for calretinin. The calretinin staining is extensively nuclear (and near-nucleolar) (Table 2; Figs. 1D–F and 2D–F).

Table 2
Quantitative analysis of double-labeling studies for the $P2X_2$ and $P2X_3$ receptors with neuronal nitric oxide synthase (NOS), calretinin, vasoactive intestinal peptide (VIP) and calcitonin gene-related peptide (CGRP) in the ganglia of the guinea pig gallbladder

	$P2X_2$-IR neurons containing NOS, calretinin, VIP or CGRP	$P2X_3$-IR neurons containing NOS, calretinin, VIP or CGRP
NOS (*n*)	58.1 ± 4% (192/332)	54.3 ± 3% (201/369)
Calretinin (*n*)	92 ± 6% (298/325)	94 ± 5% (336/358)
VIP (*n*)	30.5 ± 4% (104/341)	32.6 ± 5% (109/337)
CGRP (*n*)	0 (0/316)	0 (0/321)

n, number of double-labelled cells and the total number of cells counted, respectively, for each combination of receptor.

Double-labelling immunohistochemistry for $P2X_2$ or $P2X_3$ and VIP, showed that 30.5% of $P2X_2$ and 32.6% of $P2X_3$-immunoreactive neurons were also immunoreactive for VIP (Table 2; Figs. 1G–I and 2G–I).

Similar experiments investigating $P2X_2$ or $P2X_3$- and CGRP-immunoreactivity showed that a few CGRP-immunoreactive fibers were also immunoreactive for $P2X_2$ or $P2X_3$, but many CGRP-immunoreactive fibers outside of the ganglionated plexus were $P2X_2$- or $P2X_3$-negative (Figs. 1J–L and 2J–L).

4. Discussion

ATP and P2X receptors are known to be involved in visceral and cutaneous sensory pathways (Burnstock, 2001). The afferent nerve terminals in these pathways express $P2X_2$ and $P2X_3$ receptors and respond to application of ATP or its analogs (Bland-Ward and Humphrey, 2000). Purinergic synaptic transmission has been demonstrated in the central, peripheral, and enteric nervous systems (see Burnstock, 2003b). This has been demonstrated convincingly in the enteric nervous system in which ATP-mediated fast EPSPs have been described (Galligan and Bertrand, 1994) and characterized (LePard and Galligan, 1999) and a role in reflexes defined (Bian et al., 2000). However, no studies have been conducted to evaluate the actions of P2X receptors in the gallbladder. In the present study, we provide anatomical evidence that $P2X_2$ and $P2X_3$ receptor were expressed in the guinea pig gallbladder.

Many studies have demonstrated that extrinsic nerves pass into the gallbladder in nerve bundles that follow the blood vessels, with sympathetic fibers passing in the peri- and paravascular plexuses. Vagal preganglionic nerves and sensory fibers pass along the paravascular plexus, which consists of nerve bundles that pass parallel to the blood vessels (Mawe and Gershon, 1989; Talmage et al., 1996; Mawe et al., 1997). In the guinea pig, high levels of $P2X_2$ and $P2X_3$ expression are found in a subpopulation of

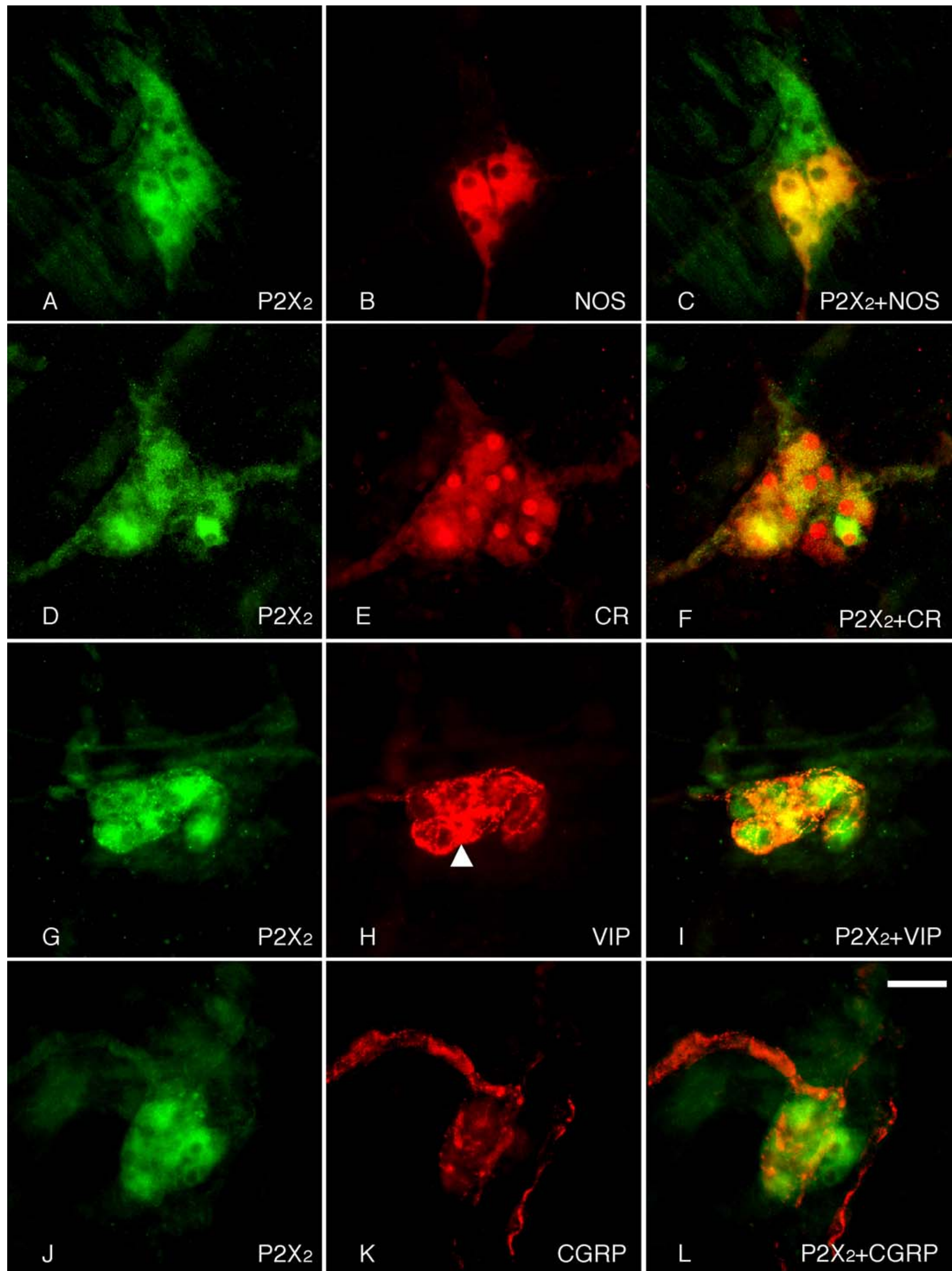

Fig. 2. Colocalization (yellow/orange) of $P2X_2$ receptor immunoreactivity with various neurochemical markers in the ganglia of the guinea pig gallbladder. $P2X_2$ receptor immunoreactivity is labeled with green FITC and the neurochemical markers with red Cy3. (A–C) Photomicrographs of the same field demonstrating the neurons that are immunoreactive for both $P2X_2$ and nitric oxide synthase (NOS). (D–F) Photomicrographs of the same field demonstrating the neurons that are immunoreactive for both $P2X_2$ and calretinin. (G–I) Photomicrographs of matching fields demonstrating neurons and nerve fibers that are immunoreactive for both $P2X_2$ and vasoactive intestinal peptide (VIP). The arrowhead indicates a neurone immunopositive for VIP in H. (J–L) Photomicrographs of the same fields demonstrating some nerve fibers that are immunoreactive for both $P2X_2$ and calcitonin gene-related peptide (CGRP; yellow/orange). Scale bars, 50 μm.

primary afferent neurons, including those of dorsal root ganglia (DRG) and nodose ganglia (see Dunn et al., 2001). Although it is not yet possible to distinguish sensory fibers that arise from spinal ganglia versus those from nodose ganglia, CGRP-containing nerve fibers are abundant in ganglia, interganglionic fiber bundles, and in the paravascular plexus (Goehler et al., 1988; Mawe and Gershon, 1989). In this study, we first processed whole mount

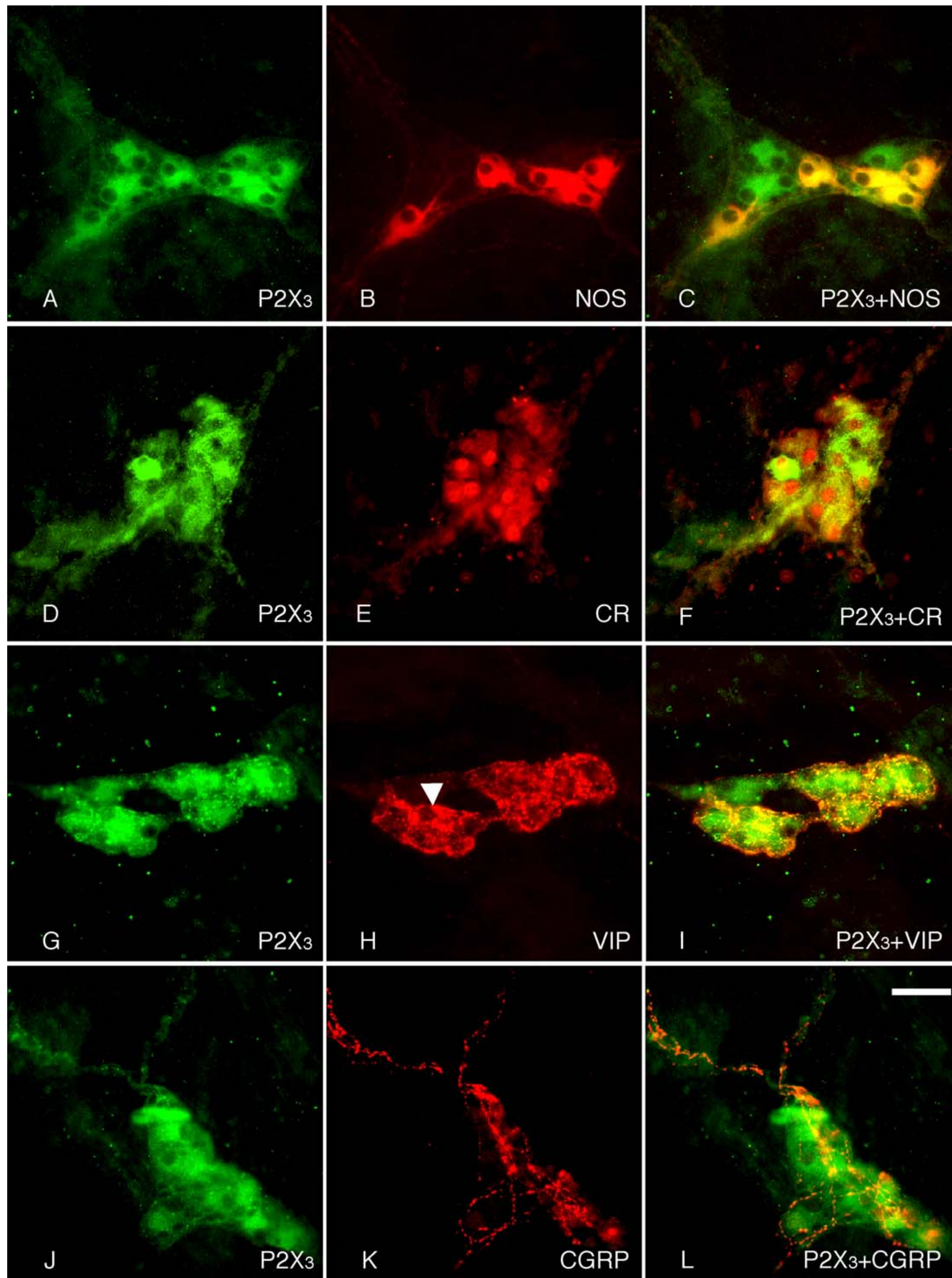

Fig. 3. Colocalization (yellow/orange) of $P2X_3$ receptor immunoreactivity with various neurochemical markers in the ganglia of the guinea pig gallbladder. $P2X_3$ receptor immunoreactivity is labeled with green FITC and the neurochemical markers with red Cy3. (A–C) Photomicrographs of the same field demonstrating the neurons that are immunoreactive for both $P2X_3$ and nitric oxide synthase (NOS). (D–F) Photomicrographs of the same field demonstrating the neurons that are immunoreactive for both $P2X_3$ and calretinin. (G–I) Photomicrographs of matching fields demonstrating neurons and nerve fibers that are immunoreactive for both $P2X_3$ and vasoactive intestinal peptide (VIP). The arrowhead indicates a neurone immunopositive for VIP in H. (J–L) Photomicrographs of the same fields demonstrating some nerve fibers that are immunoreactive for both $P2X_3$ and calcitonin gene-related peptide (CGRP). Scale bars, 50 μm.

preparations for $P2X_2$ and $P2X_3$ receptor immunoreactivity in guinea pig gallbladder. We found that $P2X_2$ and $P2X_3$ receptor-immunoreactive nerve fibers were abundant in the paravascular plexus. These nerve fibers were also immunoreactive for CGRP, suggesting that they might be sensory fibers. The $P2X_2$ and $P2X_3$-positive fibers can be followed along blood vessels and they can be seen passing through nerve bundles that link the paravascular plexus with the

ganglionated plexus. Within the ganglia, the $P2X_2$ or $P2X_3$/CGRP-positive fibers can be observed, but they are outnumbered, and in some regions obscured, by $P2X_2$ or $P2X_3$-positive, CGRP-negative nerve fibers.

Acetylcholine has long been thought to be an important excitatory neurotransmitter released from gallbladder neurons. It acts on muscarinic receptors to contract gallbladder smooth muscle (Ryan, 1987), and it acts on neuronal nicotinic receptors to mediate interganglionic communication in the gallbladder (Mawe, 1990; Mawe et al., 1994). In gallbladder ganglia, all of the neurons are cholinergic, since all are immunoreactive for the essential biosynthetic enzyme for acetylcholine, ChAT (Talmage et al., 1996). Here, we report that gallbladder neurons are also immunoreactive for $P2X_2$ and $P2X_3$. Earlier immunocytochemical studies have demonstrated that calretinin-immunoreactivity is associated with a population of cholinergic filamentous neurons (Brookes et al., 1991; Brookes, 2001). In our studies, we found that $P2X_2$ and $P2X_3$-immunoreactive neurons were also immunoreactive for calretinin.

In guinea pig, the overall population of cholinergic principal cells can be divided into two distinct subpopulations based on chemical coding patterns (Talmage et al., 1992; Talmage and Mawe, 1993). The majority of the neurons express immunoreactivities for choline acetyltransferase, substance P, neuropeptide Y, and somatostatin, and a separate group of neurons express ChAT, NOS, and VIP immunoreactivities. The issue of whether these represent two sets of neurons with different targets, or neurons with divergent inputs to the same targets, remains to be resolved. The existence of an intrinsic VIPergic innervation is a feature that is consistently encountered throughout the enteric nervous systems of mammalian species (Furness and Costa, 1987). This feature of gut innervation is apparently shared by the gallbladder where immunoreactivity for VIP is expressed at some level in gallbladder neurons and in nerve fibers (Talmage et al., 1996; Mawe et al., 1997). The $P2X_2$ and $P2X_3$-positive nerve fibers, that are extremely abundant in the gallbladder ganglia, are also immunoreactive for VIP.

It is clear that gallbladder ganglia contain neurons that express the enzymatic machinery to synthesize nitric oxide. In many species, neurons in the gallbladder express NOS and NADPH-diaphorase activity (Talmage and Mawe, 1993; Mawe et al., 1997). Nitric oxide can relax the gallbladder (Mourelle et al., 1993; McKirdy et al., 1994) as does CGRP (Kline and Pang, 1994). We found that $P2X_2$ and $P2X_3$-immunoreactive neurons were NOS-positive.

The results reported here enhance our appreciation of the neurotransmitter diversity in the wall of the gallbladder. In addition to neuroactive compounds that were previously shown to exist in the guinea pig gallbladder wall, it is now clear that intrinsic and extrinsic nerves of the gallbladder are immunoreactive for $P2X_2$ and $P2X_3$ receptors. $P2X_2$ or $P2X_3$ immunoreactivity is also expressed by most gallbladder neurons that are immunoreactive for NOS, calretinin and VIP. These results further demonstrate the complexity of the neural elements of the gallbladder, and will hopefully provoke functional studies to evaluate the actions of these neuroactive compounds in gallbladder regulation.

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