Journal of Neurochemistry, 2001, **79**, 997–1003

$P2X_2$, $P2X_{2-2}$ and $P2X_5$ receptor subunit expression and function in rat thoracolumbar sympathetic neurons

H. Schädlich,* K. Wirkner,* H. Franke,* S. Bauer,* J. Grosche,† G. Burnstock,‡ A. Reichenbach,† P. Illes* and C. Allgaier*

**Rudolf-Boehm-Institute of Pharmacology and Toxicology and †Paul-Flechsig-Institute of Brain Research, Department of Neurophysiology University of Leipzig, Leipzig, Germany*

‡*Autonomic Neuroscience Institute, London, UK*

Abstract

The present study investigated the pharmacological properties of excitatory P2X receptors and $P2X_2$ and $P2X_5$ receptor subunit expression in rat-cultured thoracolumbar sympathetic neurons. In patch-clamp recordings, ATP (3–1000 μM; applied for 1 s) induced inward currents in a concentration-dependent manner. Pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS; 30 μM) counteracted the ATP response. In contrast to ATP, α,β-meATP (30 μM; for 1 s) was virtually ineffective. Prolonged application of ATP (100 μM; 10 s) induced receptor desensitization in a significant proportion of sympathetic neurons in a manner typical for $P2X_{2-2}$ splice variant-mediated responses. Using single-cell RT-PCR, $P2X_2$, $P2X_{2-2}$ and $P2X_5$ mRNA expression was detectable in individual tyrosine hydroxylase-positive neurons; coexpression of both $P2X_2$ isoforms was not observed. Laser scanning microscopy revealed both $P2X_2$ and $P2X_5$ immunoreactivity in virtually every TH-positive neuron. $P2X_2$ immunoreactivity was largely distributed over the cell body, whereas $P2X_5$ immunoreactivity was most distinctly located close to the nucleus. In summary, the present study demonstrates the expression of $P2X_2$, $P2X_{2-2}$ and $P2X_5$ receptor subunits in rat thoracolumbar neurons. The functional data in conjunction with a preferential membranous localization of $P2X_2$/$P2X_{2-2}$ compared with $P2X_5$ suggest that the excitatory P2X responses are mediated by $P2X_2$ and $P2X_{2-2}$ receptors. Apparently there exist two types of $P2X_2$ receptor-bearing sympathetic neurons: one major population expressing the unspliced isoform and another minor population expressing the $P2X_{2-2}$ splice variant.

Keywords: ATP, excitatory P2X receptors, immunocytochemistry, $P2X_2$ isoforms, $P2X_5$, patch-clamp recordings, rat thoracolumbar sympathetic neurons, single-cell RT-PCR.

J. Neurochem. (2001) **79**, 997–1003.

ATP plays an important role as excitatory neurotransmitter/cotransmitter in both peripheral and central neurons (Illes *et al*. 1994, 2000; Kidd *et al*. 1995; Collo *et al*. 1996; Allgaier and Illes 1998). Fast synaptic responses to ATP are mediated by P2X receptors comprising a family of at least seven receptor subunits (Fredholm *et al*. 1994; Buell *et al*. 1996; Soto *et al*. 1997; Ralevic and Burnstock 1998; North and Surprenant 2000). Functional P2X receptors are intrinsic cation-selective channels permeable to Na^+, K^+ and Ca^{2+}. Native receptors may occur as homopolymers or heteropolymers, potentially of trimeric structure (Nicke *et al*. 1998).

In cultured thoracolumbar sympathetic neurons, application of ATP or 2-methylthio-ATP induces an influx of cations followed by cell membrane depolarization, the generation of action-potentials, calcium influx through voltage-sensitive Ca^{2+} channels and finally noradrenaline release (Boehm 1994; Allgaier *et al*. 1995a; Khakh *et al*. 1995; von Kügelgen *et al*. 1997; Nörenberg *et al*. 1999). The agonist α,β-methylene ATP (α,β-meATP) is considerably less effective than ATP or 2-methylthio-ATP. The ATP-induced responses are sensitive to low micromolar concentrations of the P2-receptor antagonists, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate (PPADS) and suramin, and reveal

Received July 17, 2001; revised manuscript received August 31, 2001; accepted September 14, 2001.

Address correspondence and reprint requests to Dr Clemens Allgaier, Rudolf-Boehm-Institute of Pharmacology and Toxicology, University of Leipzig, Härtelstraße 16–18, D-04107 Leipzig, Germany.
E-mail: allgc@medizin.uni-leipzig.de

Abbreviations used: FCS, fetal calf serum; α,β-meATP, α,β-methylene ATP; HBSS, Hanks' balanced salt solution; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate; TBS, Tris-buffered solution; TH, tyrosine hydroxylase.

little desensitization. These pharmacological properties suggest that the somatic receptor mediating the ATP responses in thoracolumbar neurons belongs to the $P2X_2$ or $P2X_5$ subtype (Nörenberg *et al*. 1999). Homomeric $P2X_2$ receptors are widely expressed in the CNS as well as in the peripheral nervous system, whereas expression of $P2X_5$ is much more restricted (Khakh *et al*. 2001).

The aim of the present investigation was to complete the electrophysiological characterization of excitatory P2X receptors of rat thoracolumbar sympathetic neurons with data on $P2X_2$/$P2X_5$ mRNA and protein expression using single-cell RT-PCR and confocal laser-scanning microscopy, respectively.

Materials and methods

Cultures of rat dissociated thoracolumbar ganglia

One day-old Wistar rat pups (own bred) were killed by cervical dislocation in accordance with the law of experimental animal protection under the approval of the local Committe on Animal Care and Use. Thoracolumbar paravertebral ganglia were dissected out and collected in ice-cold Hanks' balanced salt solution (HBSS) (Gibco BRL, 14170–088). After enzymatic dissociation at 37°C using collagenase (0.5 mg/mL; C 9891; Sigma, St Louis, MO, USA)/dispase II (1 mg/mL; Roche, 165859) for 20 min and trypsin (0.0625%; Gibco BRL, 25050–014) for additional 15 min, 1 mL of medium I (DMEM/F_{12} 1 : 1; Gibco, 21331–020 supplemented with 5% fetal calf serum (FCS; Seromed, S 0115), D-glucose (3 mg/mL), L-glutamine (2.5 mM; Sigma, G-7029), gentamicin (50 mg/L; Gibco BRL, 15750–037), and HEPES (15 mM), adjusted with NaOH to pH 7.2–7.4 was added and the ganglia were gently triturated with a fire-polished pipette. The cell suspension was passed through a nylon cell strainer (Falcon; 2350), and the filtrate was centrifuged at 194 *g* for 10 min at room temperature (20°C). The pellet was resuspended in medium I additionally supplemented with 0.01 mg/mL insulin (Sigma, I-6634) and 0.03 μg/mL nerve growth factor (NGF-7S; Sigma, N-0513) (referred to as medium II in the following). The number of cells was determined and adjusted to approximately 500 000 per mL as previously described for embryonic chick paravertebral ganglia (Allgaier *et al*. 1994). One hundred microlitre aliquots were plated in the centre of 35-mm culture dishes (Greiner, 627160) coated with poly-L-lysine (25 μg/mL; Sigma, P-9155), and after another 60 min 2 mL of medium II were added. The cultures were kept in an atmosphere of 5% CO_2/95% air at 37°C for 3 days (3 days *in vitro*, DIV3).

Electrophysiology

Membrane currents were recorded in the whole-cell configuration of the patch-clamp method (Hamill *et al*. 1981) at room temperature using an Axopatch 200 B (Axon Instruments) amplifier. The bath solution contained (in mM): NaCl 135; KCl 4.5; $MgCl_2$ 2, $CaCl_2$ 2; HEPES 10; glucose 10; tetrodotoxin, 0.0005; pH 7.4 adjusted with NaOH. Gigaohm seals were formed using electrodes with tip resistances of 3–5 MΩ. The internal solution consisted of (in mM): KCl 140; $MgCl_2$ 2; $CaCl_2$ 1; HEPES 10; EGTA 11; pH 7.4. The recordings were made at a holding potential of −70 mV. Drugs were applied by a pressurized fast-flow superfusion system (DAD-12; Adams and List) for 1 s each (ATP for 10 s in some instances) separated by drug-free intervals of 90 s. The solution exchange rate around the cells was below 100 ms. Data analysis was performed computer-controlled using pClamp 6.0 software (Axon Instruments). Concentration-response curves were generated in a cumulative manner and fitted using the following 3 parametric logistic function (Origin; Microcal ™ Software Inc.):

$$I = I_{max}/[1 + (EC_{50}/\text{agonist})^n],$$

where I is the steady state current produced by the agonist; I_{max} the maximal current at infinite agonist concentrations; n is the Hill coefficient, and EC_{50} is the concentration of agonist producing 50% of I_{max}.

Molecular biology

After recording, the cytoplasm of cells responding to ATP and acetylcholine was harvested under visual control (Axiovert Microscope, Zeiss) into 6 μL of intracellular solution by applying negative pressure. Only cells where the GΩ seal remained stable during suction were used for further steps. The content of the patch pipette was expelled into a PCR tube containing 2 μL dNTP/N_6 mix (2.5 mM dNTP/25 μM; Amersham Pharmacia), 1 μL dithiothreitol (100 mM), 0.5 μL RNase inhibitor (20 U; Stratagene), and 1 μL 5× first-strand buffer. Subsequently, 0.5 μL Superscript™-II-reverse transcriptase (Gibco BRL) were added and the resulting mix was incubated for 5 min at room temperature and for additional 60 min at 42°C. Amplification of tyrosine hydroxylase (TH), $P2X_5$, and $P2X_2$ cDNA fragments was carried out by nested hot-start PCR in final volumes of 50 and 25 μL at the first and the second round, respectively, using the following primers (final concentration: 100 nM) and conditions: (i) TH, semi-nested PCR. First round: TH up 5′-CGGGCTATGTAAACAGAATGGG-3′, TH low 5′-GATGGAGACTTTGGGAAAGGC-3′; initial denaturation (94°C for 2 min), 25 cycles of denaturation (94°C for 45 s), annealing (58°C for 75 s) and extension (72°C for 30 s), final extension at 72°C for 10 min. Second PCR with additional 45 cycles yielding a 235-bp product was performed with a nested 5′ primer (5′-CAATGACGCCAAGGAACCGC-3′); (ii) $P2X_5$ receptor subunit, nested PCR. $P2X_5$ up 5′- AGTCATCAACATTGGTTCTG-3′ and $P2X_5$ low 5′-CAGGAGACCTTCCGTGAAA-3′; initial denaturation (94°C for 2 min), 40 cycles of denaturation (94°C for 30 s), annealing (60°C for 30 s) and extension (72°C for 30 s), and a final extension at 72°C for 10 min. Second PCR yielding a 266-bp product was run with additional 45 cycles at an annealing temperature of 58°C and nested up (5′-CGACCTGGTACTTATCT ACCTC-3′) and low primers (5′-ACGTTCACAATGGCATTC-3); (iii) $P2X_2$ receptor subunit, re-amplification with the primer of the first run referred to as low 2 (5′-AAGTTGGGCCAAACCTTTG-3′) and up 4 (5′-ACCCCAAATATTCTTTCCGG-3′) yielding amplification products of 614 and 407 bp at an annealing temperature of 62°C (for 30 s) and a total of 85 cycles. The amplification of each TH and $P2X_5$ cDNA fragments was made with 1.5 units AmpliTaq® DNA Polymerase (Perkin Elmer); for $P2X_2$ cDNA amplification 1 unit of AmpliTaq Gold; (Perkin Elmer) was used. dNTPs were added at the second step at a final concentration of 200 μM. Amplification products were detected by ethidium bromide-staining subsequent to agarose gel electrophoresis (1.5%).

To verify the identity of amplified DNA fragments, the PCR

product was sequenced using BigDye Terminator chemistry (Applied Biosystems) and analyzed on a DNA sequencer ABI 377 (Applied Biosystems).

Immunocytochemistry

$P2X_2$ and $P2X_5$ immunoreactivity in cultured thoracolumbar sympathetic neurons (DIV3) was detected with polyclonal anti-$P2X_2$ (Biotrend) and anti-$P2X_5$ (Xiang *et al.* 1998) antibodies raised in rabbit against highly purified peptides corresponding to the carboxyl termini of the cloned rat P2X receptors ($P2X_2$, amino acids 457–472: QQDSTSTDPKGLAQL; $P2X_5$, amino acids 437–451: RENAIVNVKQSQILH) (Xiang *et al.* 1998). Sympathetic neurons were specifically stained with a monoclonal anti-TH antibody raised in the mouse (Roche Molecular Biochemicals).

The cultures were fixed in 4% paraformaldehyde dissolved in HBSS for 10 min at 4°C and washed twice for 5 min in HBSS. For permeabilization and blocking, the cells were pretreated with 0.5% Triton X-100 and 5% FCS in Tris-buffered solution (TBS) for 30 min at room temperature. Afterwards, the cells were incubated with a mixture of either TH antibody (1 : 700) and $P2X_2$ antibody (1 : 500) or TH antibody and $P2X_5$ antibody (1 : 300) in TBS containing 0.5% Triton X-100 for 24 h at 4°C. After washing, the cells were incubated in a mixture of secondary antibodies of Cy3-tagged goat anti-rabbit IgG (1 : 1000; Dianova, Hamburg, Germany) and FITC-(1 : 100; Dianova) or Cy2-tagged goat anti-mouse IgG (1 : 1000; Dianova, Hamburg, Germany) for 1.5 h at room temperature. After rinsing, the specimen were dehydrated and mounted in glycerol gelatine (Sigma). Immunofluorescence was studied by means of a laser scanning microscope (LSM 510, Zeiss) with an excitation wavelength of 488 nm (argon laser; Cy2; FITC) or of 543 nm (helium/neon laser; Cy3).

Statistics

All data are given as arithmetic means ± SEM. The significance of differences between concentration-response curves was determined by ANOVA followed by Student's *t*-test.

Drugs

The following drugs were used: α,β-meATP (Biotrend), ATP, acetylcholine, PPADS (Sigma). Stock solutions were made in extracellular medium.

Results

Patch-clamp recordings

Whole-cell patch-clamp recordings (holding potential: − 70 mV) were performed on rat cultured thoracolumbar sympathetic neurons (DIV3) exhibiting a characteristic response to acetylcholine. At 100 μM, acetylcholine-induced inward currents peaked at 890 ± 145 pA ($n = 42$).

Each of the acetylcholine-sensitive cells responded to ATP (applied for 1 s each at 90-s intervals) which induced inward currents increasing in a concentration-dependent manner (3–1000 μM) (Fig. 1a). I_{max}, EC_{50}, and Hill coefficient were 271.2 pA, 47.2 μM, and 1.6, respectively. One second after application, the response to ATP (100 μM) was diminished to 81 ± 6% of the peak current ($n = 6$). The P2 receptor antagonist PPADS (30 μM) counteracted the ATP response (Fig. 1a); the corresponding I_{max}, EC_{50}, and Hill coefficient were 169.5 pA, 45.1 μM, and 1.2, respectively. The effect of PPADS was only partially reversible after superfusion with antagonist-free medium for 10 min (Fig. 1a, inset). In contrast to ATP, α,β-meATP (30 μM; for 1 s) was virtually ineffective in about 90% of the neurons investigated (14.6 ± 3.5 pA; $n = 37$).

Prolonged application of ATP for 10 s induced receptor desensitization in three out of 13 sympathetic neurons in a manner typical for $P2X_{2-2}$ splice variant-mediated responses (Fig. 1b). At the end of the application period, currents elicited by ATP were 25.6 ± 0.8% and 81.2 ± 1.7% ($p < 0.01$) of the peak current in three and 10 neurons, respectively.

Single-cell RT-PCR

Expression of mRNA encoding $P2X_2$ and $P2X_5$ receptor subunits was investigated in cultured neurons at DIV3 with single-cell RT-PCR using subtype specific primers. Primers

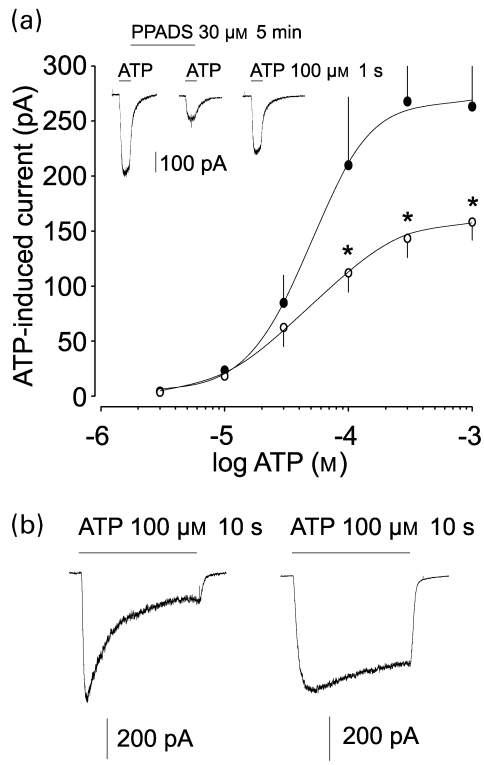

Fig. 1 (a) Concentration-response curve of ATP (3–1000 μM) in rat cultured thoracolumbar sympathetic neurons in the absence (●, $n = 5–8$) and presence (○, $n = 6–8$) of PPADS (30 μM; administered 5 min prior to and during the second ATP application). *$p < 0.05$ vs. ATP without PPADS. Inset: Representative recordings. (b) Inward currents elicited by ATP (100 μM) applied for 10 s. Representative recordings from cells showing (left; $n = 3$) or lacking (right; $n = 10$) receptor desensitization. τ_d for desensitizing currents was 2.2 ± 0.3 s ($n = 3$). For each type of neuron, ATP responses were reproducible when applied at 90-s intervals.

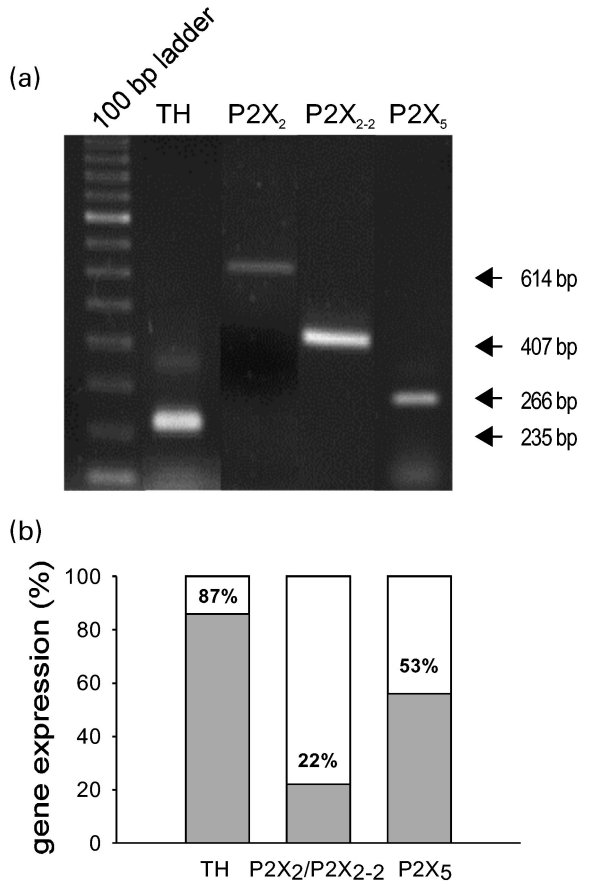

Fig. 2 (a) Single-cell RT-PCR. Representative agarose gel electrophoresis (1.5%) with ethidium bromide-stained cDNA fragments: TH, 235 bp; $P2X_2$, 614 bp; $P2X_{2-2}$, 407 bp; $P2X_5$, 266 bp. (b) Proportion (%) of individual neurons expressing TH, $P2X_2$ or $P2X_5$ mRNA (number of harvested cells: TH, 15; $P2X_2$, 27; $P2X_5$, 19).

selective for TH cDNA fragments were used as a positive control as well as in order to verify the sympathetic phenotype of the investigated cells. Prior to harvesting the cytoplasm, inward currents evoked by successive application of acetylcholine (100 μM; 1 s) and ATP (100 μM; 1 s) were recorded in the whole-cell patch-clamp configuration. Only cells exhibiting regular responses to each agonist were used for subsequent RT-PCR. The amplification products were analysed by agarose gel electrophoresis.

In 13 out of 15 neurons (87%), mRNA coding for TH was detected by the occurrence of a 235-bp amplification product (Fig. 2a). The $P2X_2$ primers generated both a 614-bp $P2X_2$ cDNA fragment (two out of 27 cells) and a 407-bp amplification product representing the $P2X_{2-2}$ splice variant (four out of 27 cells) with a 207-bp deletion in the C-terminal domain (Fig. 2b). Thus, mRNA encoding $P2X_2$/$P2X_{2-2}$ cDNA fragments was detected in 22% of the cells investigated. Co-expression of both $P2X_2$ receptor isoforms was not observed in any of the individual cells. mRNA coding for $P2X_5$ cDNA fragments was detected in 10 out of 19 cells (53%) (Fig. 2b). The identity with the known $P2X_2$, $P2X_{2-2}$, and $P2X_5$ genes was verified by sequence analysis.

Immunocytochemistry

Cultured thoracolumbar sympathetic neurons (DIV3) were studied with antibodies specific for $P2X_2$ or $P2X_5$ receptor subunits and for TH (Fig. 3). Virtually all cells exhibiting TH immunoreactivity showed additionally $P2X_2$ or $P2X_5$ immunoreactivity in double staining experiments. $P2X_2$ immunoreactivity was largely distributed over the cell body, whereas $P2X_5$ immunoreactivity was most distinctly located close to the nucleus. No specific staining was found in negative controls (without primary antibody, or with the primary antibody pre-incubated with antigen).

Discussion

The functional properties of excitatory P2X receptors have been investigated in a variety of sympathetic neurons (Dunn *et al.* 2001) including guinea-pig coeliac ganglia (Silinsky and Gerzanich 1993; Khakh *et al.* 1995), rat superior cervical ganglia (Khakh *et al.* 1995; Boehm 1999), and chick and rat thoracolumbar ganglia (Allgaier *et al.* 1995a,b; Nörenberg *et al.* 1999). The P2X receptors of these sympathetic neurons exhibit some common features that were corroborated by the present patch-clamp studies on rat thoracolumbar sympathetic neurons: (i) ATP induced inward currents at low micromolar concentrations with a maximum effect of 300 μM and a peak response that decayed by only 20% during a 1-s application period; (ii) PPADS, at low micromolar concentrations antagonized the response to ATP; and (iii) α,β-meATP (30 μM) was virtually ineffective. Accordingly, the excitatory P2X receptors in rat thoracolumbar neurons exhibited pharmacological properties similar to those of homomeric $P2X_2$ and $P2X_5$ receptors (Collo *et al.* 1996; Nörenberg *et al.* 1999; North and Surprenant 2000).

$P2X_2$ mRNA expression or $P2X_2$ immunoreactivity has been detected throughout the brain and spinal cord, as well as in sensory and autonomic ganglia including the dorsal root ganglion, superior cervical ganglion, and coeliac ganglion (Xiang *et al.* 1998). In contrast, the expression pattern of the $P2X_5$ receptor subunit is much more restricted. In the CNS, $P2X_5$ mRNA was only found in the mesencephalic nucleus of the trigeminal nerve at which propioceptive primary afferents from muscles of mastication terminate, and in motoneurons of the spinal cord ventral horn (Collo *et al.* 1996). In the peripheral nervous system, $P2X_5$ mRNA expression or $P2X_5$ immunoreactivity was detected in sensory ganglia (Xiang *et al.* 1998), pelvic ganglia (Zhong *et al.* 1998), cultured superior cervical ganglia (Li *et al.* 2000), and the spiral ganglion of the inner

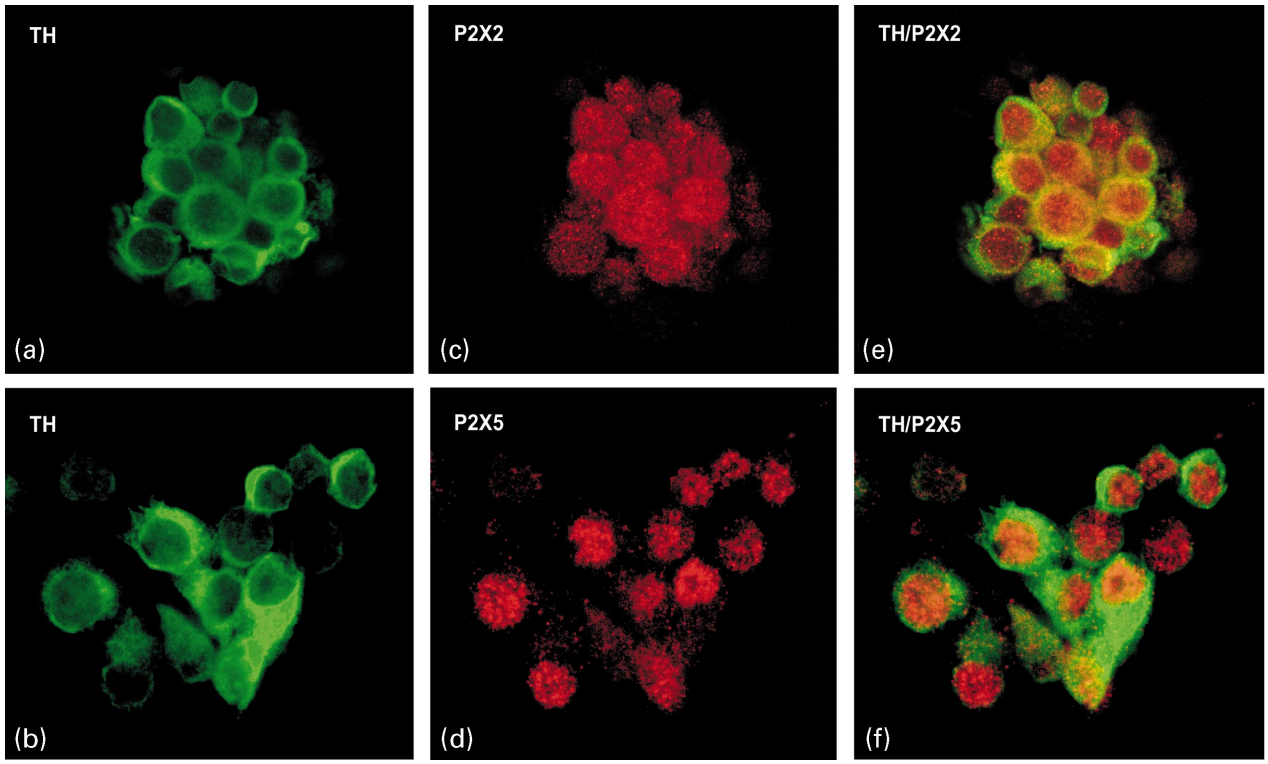

Fig. 3 Immunocytochemistry for tyrosine hydroxylase (TH) as well as for $P2X_2$ and $P2X_5$ receptor subunits in rat cultured thoracolumbar sympathetic neurons. Individual cell cultures were studied either with primary antibodies against TH and the $P2X_2$ receptor subunit (a, c and e) or TH and the $P2X_5$ receptor subunit (b, d and f) (magnification: 500-fold).

ear (Xiang *et al*. 1999), but not in tissue sections of superior cervical ganglia or coeliac ganglia (Xiang *et al*. 1998).

The present immunocytochemical experiments on rat cultured thoracolumbar sympathetic ganglia demonstrated the expression of both the $P2X_2$ and the $P2X_5$ receptor subunit in virtually every TH-positive neuron (Fig. 3). The RT-PCR approach additionally detected both the full-length $P2X_2$ isoform and a splice variant with a 207-bp deletion in the C-terminal domain ($P2X_{2-2}$), but no coexpression of either isoform in any of the neurons investigated (Fig. 2). In contrast to immunocytochemistry, single-cell RT-PCR was successful only in a smaller number of sympathetic neurons. The apparent discrepancy with the immunocytochemistry data could be explained by a lack of linear correlation between the mRNA content of the cell and the amount of protein translated. However, degradation of $P2X_2$ mRNA during harvesting or other technical reasons may also account for the low efficiency of $P2X_2$ amplification by single-cell RT-PCR, considering that about 10^2 cDNA molecules are initially required to obtain detectable amplification products using RT-PCR (Brändle *et al*. 1999).

In particular, two observations favour the idea that excitatory ATP responses in sympathetic neurons were mediated by homomeric $P2X_2$ rather than by homomeric $P2X_5$ receptors: (i) the activation of recombinant $P2X_5$ receptors has been shown to induce only very small currents, at least two orders of magnitude below those observed for $P2X_2$ as well as for $P2X_1$, $P2X_3$ and $P2X_4$ (North and Surprenant 2000), and (ii) $P2X_2$ immunoreactivity was detected in the membrane, whereas $P2X_5$ immunoreactivity was expressed at a much lower level and was predominantly located close to the nucleus (Fig. 3). Accordingly, also the formation of $P2X_{2/5}$ heteromers appears unlikely. However, since $P2X_{2/5}$ coimmunoprecipitation has been demonstrated on transfected HEK 293 cells (Torres *et al*. 1999), $P2X_{2/5}$ receptor expression cannot be excluded in sympathetic neurons until double-labelling immunofluorescence experiments with $P2X_2$ and $P2X_5$ antibodies are performed.

A nuclear location of P2X immunoreactivtiy has previously been observed, e.g. with $P2X_7$ antibodies on dissociated Müller cells (Pannicke *et al*. 2000). Additional studies are required to elucidate the functional significance of nuclear P2X receptors. In neurosecretory neurons of the supraoptic nucleus, $P2X_2$ immunoreactivity was predominantly associated with the endoplasmatic reticulum suggesting the involvement of $P2X_2$ receptors in the regulation of Ca^{2+} release from intracellular stores (Loesch *et al*. 1999).

Homomeric receptors of both $P2X_2$ isoforms exhibit similar pharmacological properties (Brändle *et al*. 1997;

Simon *et al*. 1997). In Xenopus oocytes and HEK293 cells, the responses of recombinant $P2X_2$ or $P2X_{2-2}$ receptors to ATP were of similar magnitude, and both receptor subtypes were virtually insensitive to α,β-meATP. In addition, the ATP-induced currents were highly sensitive to PPADS and suramin in both cases. However, after a prolonged application of ATP (10 s), $P2X_{2-2}$ receptors desensitized faster and more strongly than $P2X_2$ receptors (Brändle *et al*. 1997; Simon *et al*. 1997). Thus, the currents recorded upon a 10-s application of ATP in the present study (Fig. 1b) provide functional evidence for the occurrence of two distinct types of excitatory P2X receptor-bearing thoracolumbar sympathetic neurons; (i) one major population expressing the unspliced $P2X_2$ isoform, and (ii) a minor population expressing the $P2X_{2-2}$ splice variant. Future studies will be devoted to an elucidation of the functional implications of this neuronal diversity.

Acknowledgements

The present study was supported by the Deutsche Forschungsgemeinschaft (AL 414/1–3) and Interdisciplinary Centre for Clinical Research at The University of Leipzig (01KS9504, Project Z10). The authors wish to express their gratitude to Helga Sobottka for skillful technical assistance, Dr Thomas Wheeler-Schilling (Augenklinik, Universität Tübingen) for helpful discussion, and Dr Knut Krohn (IZKF, Universität Leipzig) for DNA sequencing.

References

Allgaier C. and Illes P. (1998) Neuromodulation in the cardiovascular system, in *Cardiovascular Biology of Purines* (Burnstock, G., Dobson, J. G., Liang, B. T. and Linden, J., eds), pp. 257–270. Kluwer Academic Publishers, Dordrecht/Boston/London.

Allgaier C., Schobert A., Belledin M., Jackisch R. and Hertting G. (1994) Modulation of electrically evoked [^{3}H]-noradrenaline release from cultured chick sympathetic neurones. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **350**, 258–266.

Allgaier C., Wellmann H., Schobert A., Kurz G. and von Kügelgen I. (1995a) Cultured chick sympathetic neurones: ATP-induced noradrenaline release and its blockade by nicotinic receptor antagonists. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **352**, 25–30.

Allgaier C., Wellmann H., Schobert A. and von Kügelgen I. (1995b) Cultured chick sympathetic neurones: modulation of electrically evoked noradrenaline release by P2-purinoceptors. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **352**, 17–24.

Boehm S. (1994) Noradrenaline release from rat sympathetic neurones evoked by P_2-purinoceptor activation. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **350**, 454–458.

Boehm S. (1999) ATP stimulates sympathetic transmitter release via presynaptic P2X purinoceptors. *J. Neurosci.* **19**, 737–746.

Brändle U., Spielmanns P., Osteroth R., Sim J., Surprenant A., Buell G., Ruppersberg J. P., Plinkert P. K., Zenner H.-P. and Glowatzki E. (1997) Desensitization of the $P2X_2$ receptor controlled by alternative splicing. *FEBS Lett.* **404**, 294–298.

Brändle U., Zenner H.-P. and Ruppersberg J. P. (1999) Gene expression of P2X-receptors in the developing inner ear of the rat. *Neurosci. Lett.* **273**, 105–108.

Buell G., Collo G. and Rassendren F. (1996) P2X receptors: an emerging channel family. *Eur. J. Neurosci.* **8**, 2221–2228.

Collo G., North R. A., Kawashima E., Merlo-Pich E., Neidhart S., Surprenant A. and 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 P. M., Zhong Y. and Burnstock G. (2001) P2X receptors in peripheral neurons. *Prog. Neurobiol.* **65**, 107–134.

Fredholm B. B., Abbracchio M. P., Burnstock G., Daly J. W., Harden T. K., Jacobson K. A., Leff P. and Williams M. (1994) Nomenclature and classification of purinoceptors. *Pharmacol. Rev.* **46**, 143–156.

Hamill O. P., Marty A. G., Neher E., Sakman B. and Sigworth F. J. (1981) Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Arch.* **391**, 85–100.

Illes P., Sevcik J., Finta E. P., Fröhlich R., Nieber K. and Nörenberg W. (1994) Modulation of locus coeruleus neurons by extra- and intracellular adenosine 5′-triphosphate. *Brain Res. Bull.* **35**, 513–519.

Illes P., Klotz K.-N. and Lohse M. J. (2000) Signaling by extracellular nucleotides and nucleosides. *Naunyn-Schmiedeberg's Arch. Pharmacol.* **362**, 295–298.

Khakh B. S., Humphrey P. P. A. and Suprenant A. (1995) Electrophysiological properties of P2X-purinoceptors in rat superior cervical, nodose and guinea-pig coeliac neurones. *J. Physiol.* **484**, 385–395.

Khakh B. S., Burnstock G., Kennedy C., King B. F., North R. A., Seguela P., Voigt M. and Humphrey P. P. (2001) International Union of Pharmacology. XXIV. Current status of the nomenclature and properties of P2X receptors and their subunits. *Pharmacol. Rev.* **53**, 107–118.

Kidd E. J., Grahames B. A., Simon J., Michel A. D., Barnard E. A. and Humphrey P. P. A. (1995) Localization of P_{2X} purinoceptor transcripts in the rat nervous system. *Mol. Pharmacol.* **48**, 569–573.

von Kügelgen I., Nörenberg W., Illes P., Schobert A. and Starke K. (1997) Differences in the mode of stimulation of cultured rat sympathetic neurons between ATP and UDP. *Neuroscience* **78**, 935–941.

Li G.-H., Lee E. M., Blair D., Holding C., Poronnik P., Cook D. I., Barden J. A. and Bennett M. R. (2000) The distribution of P2X receptor clusters on individual neurons in sympathetic ganglia and their redistribution on agonist activation. *J. Biol. Chem.* **37**, 29107–29112.

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

Nicke A., Bäumert H. G., Rettinger J., Eichele A., Lambrecht G., Mutschler E. and Schmalzing G. (1998) $P2X_1$ ad $P2X_3$ receptors form stable trimers: a novel structural motif of ligand-gated ion channels. *EMBO J.* **17**, 3016–3028.

Nörenberg W., von Kügelgen I., Meyer A. and Illes P. (1999) Electrophysiological analysis of P2-receptors mechanisms in rat sympathetic neurones. *Prog. Brain Res.* **120**, 209–221.

North R. A. and Surprenant A. (2000) Pharmacology of cloned P2X receptors. *Ann. Rev. Pharmacol. Toxicol.* **40**, 563–580.

Pannicke T., Fischer W., Biedermann B., Schädlich H., Stewart U., Faude F., Wiedemann P., Allgaier C., Illes P., Burnstock G. and Reichenbach A. (2000) $P2X_7$ receptors in Müller glial cells from the human retina. *J. Neurosci.* **20**, 5965–5972.

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

Silinsky E. M. and Gerzanich V. (1993) On the excitatory effects of ATP and its role as a neurotransmitter in coeliac neurons of the guinea-pig. *J. Physiol.* **464**, 197–212.

Simon J., Kidd E. J., Smith F. M., Chessel I. P., Murrell-Lagnado R., Humphrey P. P. A. and Barnard E. A. (1997) Localization and functional expression of splice variants of the $P2X_2$ receptor. *Mol. Pharmacol.* **52**, 237–248.

Soto F., Garcia-Guzman M. and Stühmer W. (1997) Cloned ligand-gated channels activated by extracellular ATP (P2X receptors). *J. Membrane Biol.* **160**, 91–100.

Torres G. E., Egan T. M. and Voigt M. M. (1999) Identification of a domain involved in ATP-gated ionotropic receptor subunit assembly. *J. Biol. Chem.* **274**, 6653–6659.

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

Xiang Z., Bo X. and Burnstock G. (1999) P2X receptor immunoreactivity in the rat cochlea, vestibular ganglion and cochlear nucleus. *Hear. Res.* **128**, 190–196.

Zhong Y., Dunn P. M., Xiang Z., Bo X. and Burnstock G. (1998) Pharmacological and molecular characterization of P2X receptors in rat pelvic ganglion neurons. *Br. J. Pharmacol.* **125**, 771–781.