British Journal of Pharmacology (2000) 131, 1775–1783

www.nature.com/bjp

Local regulation of [^{3}H]-noradrenaline release from the isolated guinea-pig right atrium by P_{2X}-receptors located on axon terminals

*,[1]Beáta Sperlágh, [2]Ferenc Erdélyi, [2]Gábor Szabó & [1]E. Sylvester Vizi

[1]Department of Pharmacology, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, H-1450 Budapest, POB 67, Hungary and [2]Laboratory of Molecular Biology and Genetics, Institute of Experimental Medicine, Hungarian Academy of Sciences, Budapest, H-1450 Budapest, POB 67, Hungary

1 In this study the regulation of cardiac sympathetic outflow by presynaptic P_{2X} receptor-gated ion channels was examined.

2 ATP (30 μM–1 mM) and other P2-receptor agonists elicited [^{3}H]-noradrenaline ([^{3}H]-NA) outflow from the isolated guinea-pig right atrium with the potency order of ATP > 2-methyl-thioATP > α,β-methylene-ATP = ADP, whereas β,γ-methylene-L-ATP was inactive.

3 Ca^{2+}-free conditions abolished both electrical field stimulation (EFS)- and ATP-evoked release of tritium. Unlike from EFS-induced outflow, ATP-induced [^{3}H]-NA outflow was not reduced by ω-Conotoxin-GVIA (100 nM), Cd^{2+} (100 μM) and tetrodotoxin (1 μM).

4 The rapid extracellular decomposition of ATP was revealed by HPLC analysis. However, the effect of ATP to promote [^{3}H]-NA release was not prevented by 8-cyclopentyl-1,3-dipropylxanthine (DPCPX, 250 nM), 3,7-dimethyl-1-propargylxanthine (DMPX, 250 nM), or by reactive blue 2 (RB2, 10 μM), antagonists of A_1-, A_2- and inhibitory P_2 receptors.

5 Zn^{2+} (50 μM), the P_{2X}-receptor modulator potentiated, and P_{2X} receptor antagonists, i.e. suramin (300 μM), pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS, 30 μM) and 2′-o-(trinitrophenyl)-adenosine 5′-triphosphate (TNP-ATP, 30 μM) antagonized the ATP (1 mM)-evoked response.

6 RT–PCR study revealed the expression of P_{2X2} and P_{2X3} receptor mRNAs in guinea-pig superior cervical ganglion.

7 PPADS (30 μM) significantly reduced the EFS-induced [^{3}H]-NA outflow in the presence DPCPX (250 nM) and RB2 (10 μM).

8 In summary a P_{2X}-type purinoceptor regulates noradrenaline release from the isolated right atrium of the guinea-pig. The pharmacological profile of the receptor resemble to homo-oligomeric P_{2X3} or hetero-oligomeric P_{2X2}/P_{2X3} complexes, and provide a new target to intervene on sympathetic neuroeffector transmission at the presynaptic site.

British Journal of Pharmacology (2000) **131,** 1775–1783

Keywords: Noradrenaline; release; P_{2X}-purinoceptors; atrium; guinea-pig; RT–PCR

Abbreviations: ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; ATP, adenosine 5′-triphosphate; DMPX, 3,7-dimethyl-1-propargylxanthine; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; EFS, electrical field stimulation; [^{3}H]-NA, [^{3}H]-noradrenaline; α,β-methylene-ATP, α,β-methylene-adenosine 5′-triphosphate; β,γ-methylene-L-ATP, β,γ-methylene-L-adenosine triphosphate; 2-methyl-thioATP, 2-methylthioadenosine triphosphate; NA, noradrenaline; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid; RB2, reactive blue 2; RT–PCR, reverse-transcription-coupled-polymerase-chain-reaction; SCG, superior cervical ganglion; TNP-ATP, 2′-o-(trinitrophenyl) adenosine 5′-triphosphate; TTX, tetrodotoxin

Introduction

Presynaptic ligand-gated ion channels provide an important target to amplify neuroeffector transmission (MacDermott *et al*., 1999). ATP gated ion channels, also known as P_{2X} receptors, form a unique family of ligand-gated ion channels, characterized by two membrane spanning regions and a large extracellular loop (Brake *et al*., 1994). Until now seven different members of this family has been identified which display distinct but overlapping distribution and pharmacological features (North & Surprenant, 2000; Ralevic & Burnstock, 1998). These seven P_{2X} proteins, numbered by P_{2X1} to P_{2X7}, however, do not function as individual receptors, but they form homo- or hetero-oligomeric complexes in expression systems, corresponding to functional ATP-gated ion channels in native tissues. Recent biochemical evidence suggest that as much as 17 different homo- or hetero-oligomeric combinations of P_{2X} receptor subtypes result in functional receptor (Torres *et al*., 1999). The coassembly of different individual P_{2X} subtypes may result in a completely novel pharmacological phenotype (Haines *et al*., 1999; Le *et al*., 1998, 1999; Lewis *et al*., 1995), but this is not necessarily the case; in other combinations one subtype dominates over the other in terms of pharmacological profile (King *et al*., 2000). Given this considerable diversity, a major challenge has recently been shown to characterize P_{2X} receptor assemblies in expression systems and to identify physiological responses by their native counterparts. In the nervous system P_{2X} receptors mediate the well-known fast transmitter action of extracellular ATP in neuro-neuronal (Edwards *et al*., 1992) and neuro-effector synapses (Evans *et al*., 1992). As P_{2X} receptors are also known for their high Ca^{2+} permeability (Rogers *et al*., 1997), whereby Ca^{2+} signal

*Author for correspondence.

and neurotransmitter release could be directly initiated, another possible function of neuronal P_{2X} receptors is that they modulate neurotransmitter release. In our earlier study we demonstrated presynaptic P_2 receptor on cholinergic nerve terminals (Sperlágh & Vizi, 1991), which was confirmed by electrophysiological studies (Sun & Stanley, 1996), and followed by reports on P_{2X} receptors regulating glutamate (Gu & MacDermott, 1997) dopamine (Zhang *et al*., 1996) and GABA release (Hugel & Schlichter, 2000). Apart from our previous study, where the presence of P_2 receptors on sympathetic nerve terminals has been first suggested (Sperlágh & Vizi, 1991), recent reports identified stimulatory P_{2X} receptors on noradrenergic axon terminals of cultured rat sympathetic neurons (Boehm, 1999, von Kugelgen *et al*., 1999). However, the organization and receptor distribution of cultured neurons might not reflect that of normal tissue and no such data is available concerning the noradrenaline release in whole tissue. Furthermore a wealth of data suggest that there is considerable inter-species and inter-ganglionic variation in the expression of P_{2X} receptors in sympathetic ganglia of different species (Evans & Surprenant, 1996; Xiang *et al*., 1998; Zhong *et al*., 2000a,b), therefore one could not extrapolate from one species to another, or from one ganglion to another.

The main question addressed in this study was, therefore, to show whether the release of noradrenaline could be modulated by presynaptic P_{2X} receptors in the isolated right atrium of the guinea-pig, and to characterize pharmacologically the receptor-subtype involved.

Methods

[^{3}H]-Noradrenaline release from the isolated right atrium of the guinea-pig

[^{3}H]-Noradrenaline release experiments were carried out according to the method described in our earlier papers (e.g. Oe *et al*., 1999). Male albino guinea-pigs (Richter Gedeon Co., Budapest, Hungary) of 300–500 g body weights were anaesthetized with saturating concentrations of diethyl-ether, and then stunned and exsanguinated. The right atria were dissected in ice-cold Krebs' solution saturated with 95% O_2 and 5% CO_2 and incubated in 1 ml of modified Krebs solution (mM: NaCl 113, KCl 4.7, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, glucose 11.5, pH 7.8) containing 370 kBq ml^{-1} [^{3}H]-NA (0.27 μM, specific activity 1.35 TBq mmol^{-1}; Amersham), ascorbic acid (30 μM) and Na_2EDTA (100 μM) for 60 min. The medium was bubbled continuously with 95% O_2 and 5% CO_2 and maintained at 37°C. After incubation the tissues were rinsed and transferred to tissue chambers of 4-ml volume and perfused continuously with modified Krebs' solution at a rate of 1 ml min^{-1}. In order to wash out the excess radioactivity and to allow tissue equilibration, the preparations were perfused for 60-min. Subsequently, perfusate samples were collected over a 3-min period and assayed for [^{3}H]-NA. During the sample collection period, electrical field stimulation was applied twice 30 min apart (S_1, S_2), using a Grass S88 stimulator (Quincy, MA, U.S.A.), with the following parameters: alternate square-wave pulses (25 V cm^{-1}, 2.5-ms duration) applied at 2 Hz; a total of 240 shocks were delivered during both S_1 and S_2. In some experiments DPCPX and RB2 were perfused during the entire collection period and PPADS was applied 18 min before S_2 and thereafter. In other experiments only one electrical stimulation (S_1) was applied, and 12 min after stimulation P_2 receptor agonists (ATP, 2-methyl-thioATP, ADP, α,β-methylene-ATP, β,γ-methylene-L-ATP) were applied for a 10 min perfusion period. Purinoceptor antagonists (PPADS, DPCPX, DMPX, suramin, TNP-ATP, RB2 and NF 279) and Zn^{2+} were administered 10 min before ATP, tetrodotoxin, Cd^{2+}, and ω-conotoxin GVIA were perfused from 12 min before S_1, Ca^{2+} free solution was applied 60 min before S_1 and thereafter.

The radioactivity released from the preparations was measured with a Packard 1900 Tricarb (Packard, Canberra, Australia) liquid scintillation spectrometer. A 0.5-ml aliquot of the perfusate sample was added to 4 ml of liquid scintillation fluid (Packard Ultima Gold) and counts were determined. For determining the residual radioactivity, the tissues were weighed and homogenized, and the radioactivity was extracted with 10% trichloroacetic acid. The counts were converted to absolute activity by the external standard method. Release of [^{3}H]-NA was expressed in Bq g^{-1} and as a percentage of the amount of radioactivity in the tissue at the sample collection time (fractional release). It was reported previously that the majority of tritium outflow under identical conditions is derived from [^{3}H]-NA (Nakatsuka *et al*., 1995). Basal outflow was calculated as the fractional release measured in a 3-min sample in the absence and presence of drugs, respectively. Drug-induced NA outflow was expressed by calculating the net release in response to drug application by the area-under-the-curve method, i.e. by subtracting the release before the drug application from the values measured after drug application. Similarly, electrical-stimulation evoked NA release was calculated by subtracting the release before the stimulation from the values measured after the respective stimulation (S_1,S_2). The effect of drugs on the electrical stimulation-evoked release of [^{3}H]-NA was expressed as S_2/S_1 ratios, measured in the absence and presence of the drug.

ATP metabolism studies

Right atria were dissected from guinea-pigs as described above, subdivided into three pieces and incubated in 3 ml of Krebs solution at 37°C, bubbled with 95% O_2 + 5% CO_2. Subsequently 60 nmol of ATP was added to the 3-ml bath and aliquots of 70 μl were taken out 2.5, 5, 10, 15, 20 and 30 min after the addition of ATP. The concentrations of ATP, ADP, AMP, adenosine and inosine in the aliquots were measured by high-performance liquid chromatography combined with ultraviolet detection (HPLC-UV) according to the method described earlier (Sperlágh *et al*., 1995). The actual concentrations of ATP, ADP, and AMP were expressed in μM. For the determination of the kinetic parameters of ectoATPase, a linear regression for ATP or AMP concentrations as a function of time was calculated from the concentrations of the first five samples (0, 2.5, 5, 10 and 15) after three different initial concentrations of ATP or AMP (20, 100 and 500 μM), and the slope was used as initial velocity (v_i). These initial velocities were used for calculation of the parameters from the Lineweaver–Burk plot using linear line regression:

$$1\ v_i^{-1} = K_M\ v_{max}^{-1} \times [S] + 1\ v_{max}^{-1},$$

where v_i is the velocity measured when very little substrate has reacted, [S] is the concentration of the substrate, V_{max} (maximal velocity) is a point where the enzyme is saturated with the substrate and K_M (Michaelis constant) is the concentration that produces half-maximal velocity.

RT–PCR study

Dissected organs (guinea-pig superior cervical ganglion, or hippocampus) were collected into liquid nitrogen. Total RNA from tissue samples was isolated by a modified guanidine isothiocyanate method with TRIZOL Reagent (Life Technologies, Rockville, MD, U.S.A.). DNA contamination of RNA preparations was eliminated by treatment with RNase free DNase (Promega, Madison, WI, U.S.A.) as described Ausubel *et al*. (1988). First-strand cDNA templates were synthesized from 5 μg of DNase treated RNA samples with SuperScript Preamplification System (Life Technologies) using random nonamer primers in 20 μl total volume and 5 μl of the reactions were applied for PCR amplification using 0.4 μM forward and reverse primers. Amplifications were carried out as follows: 5 min initial denaturation at 96°C then 2 u of Taq DNA polymerase (Promega) was added to the reactions and 35 cycles at 94°C for 1 min, 59°C for 1 min, 72°C for 1 min followed by 5 min final extension at 72°C.

Primers for amplication of guinea-pig P_{2X2} cDNA were designed to the common region of the known three splice variants (AF053327, AF053328, AF053329) (Parker *et al*., 1998) between positions 406 (AGCATCATCACCAGGATTGAG) and 897 (GATGATGACGCCAATAACACC). Oligonucleotides were synthesized by MWG Biotech AG (Ebersberg, Germany). Primers for amplification of the P_{2X3} cDNA were designed to rat P_{2X3} sequence (X91167) between positions 742 (CTCCTGCCTAACCTCACCGACAAGGACATAAAGAGGTGCCGCTTC) and 1321 (CTAGTGACCAATAGAATAGGCCCCTGAGTCTGTAGACTGCTTCTC) and were kindly provided by J. Simon. Since the guinea-pig sequence is not available, mouse β-actin primers were used for control amplification (AGCTGAGAGGGAAATCGTGC and GATGGAGGGCCGGCTCAT, positions 569 and 1048 on cDNA) (Tokunaga *et al*., 1986). Amplification products were analysed by agarose gelelectrophoresis.

Materials

The following chemicals were used: [^{3}H]-noradrenaline, ([^{3}H]-NA, Amersham, Little Chalfont, U.K.) adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), adenosine 5′-monophosphate (AMP), adenosine, inosine, tetrodotoxin (TTX), α,β-methylene-adenosine 5′-triphosphate (α,β-methylene-ATP) (all from Sigma, St. Louis, MO, U.S.A.), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), 3.7-dimethyl-1-propargylxanthine (DMPX), 2-methylthioadenosine triphosphate (2-methyl-thioATP), pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid tetrasodium (PPADS) (RBI, Natick, MA, U.S.A.), $ZnCl_2$ $CdCl_2$ (Reanal, Budapest, Hungary), ω-conotoxin GVIA (Alomone Labs, Jerusalem, Israel), suramin (Bayer, Leverkusen, Germany), 2′-o-(trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP, Molecular Probes, Eugene, OR, U.S.A.), β,γ-methylene-L-adenosine triphosphate (β,γ-methylene-L-ATP), NF 279 (Tocris Cookson, Ballwin, MO, U.S.A.), reactive blue 2 (RB2) (Aldrich, Steinheim, Germany). All solutions were freshly prepared on the day of use.

Statistics

All data were expressed as means$\pm$s.e.mean of n observations. The statistical analysis were made by one-way analysis of variance (ANOVA) followed by Dunnett test (multiple comparisons), or Student's t-test (pairwise comparisons). P values of less than 0.05 were considered statistically significant. Concentration-response curves were constructed using the sigmoidal logistic equation of the GraphPad Prism (San Diego, CA, U.S.A.) software.

Results

[^{3}H]-noradrenaline release experiments on the isolated right atrium of the guinea-pig

After 60 min preperfusion the basal tritium efflux measured in a 3 min sample was 0.32$\pm$0.02% (n=8) of tissue tritium content, which remained relatively constant during the subsequent sample collections. Electrical field stimulation of low frequency (2 Hz, 240 shocks) increased the outflow of [^{3}H]-NA: the electrical stimulation-evoked release was 1.22$\pm$0.13%, (n=8, $P<0.001$) in control experiments (Figure 1a). The second stimulation (S_2) elicited a similar amount of tritium, resulting in an S_2/S_1 ratio of 0.96$\pm$0.02 (n=8). In the presence of the sodium channel inhibitor tetrodotoxin (TTX, 1 μM) the basal outflow of [^{3}H]-NA did not change significantly (0.34$\pm$0.01, n=6, $P>0.05$); in contrast there was no detectable increase in the outflow, in response to stimulation (see Figure 2).

A 10-min perfusion of the preparations with ATP (1 mM) caused a transient increase in the efflux of [^{3}H]-NA (Figure

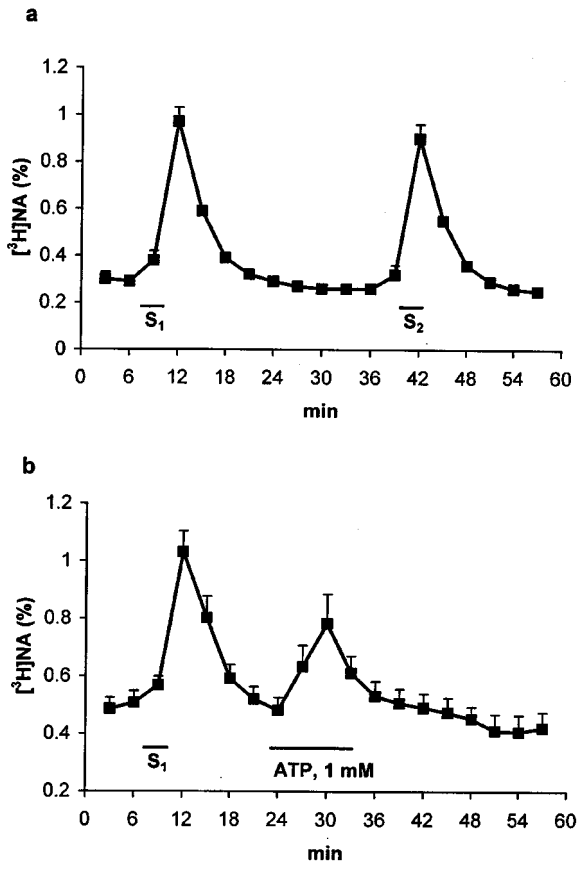

Figure 1 Electrical field stimulation- and ATP-induced release of [^{3}H]-NA from the isolated guinea-pig right atrium. Tissues were superfused with Krebs' solution for 60 min and then subjected to electrical field stimulations (a,b) S_1, S_2, 25 V cm^{-1}, 2 Hz, 2.5 ms, 240 shocks) or to ATP (1 mM) application (b) as indicated. The tritium content in the perfusate samples was measured by liquid scintillation spectrometry and was expressed in fractional release (%, for calculation, see Methods), as a function of time. Data represent the means$\pm$s.e.mean of 6–8 identical experiments.

1b) which peaked 6 min after ATP administration and then gradually declined and returned to the baseline level in the following 18 min. The net release evoked by ATP (1 mM) ($1.23 \pm 0.2\%$), was comparable to that evoked by field stimulation, and was concentration-dependent between 0.03 and 1 mM (see Figure 5). As it is known that high concentration of ATP might have non-specific depolarizing effect, higher concentration than 1 mM was not tested.

In order to explore the potential mechanism whereby ATP promotes the outflow of noradrenaline, in subsequent experiments electrical field stimulation (EFS)-induced and ATP-induced [^{3}H]-NA outflow were compared under different experimental conditions. When tissues were exposed to Ca^{2+} free solution supplemented with 1 mM EGTA both EFS- and ATP induced [^{3}H]-NA outflow was abolished (Figure 2). ω-Conotoxin-GVIA (100 nM) the selective blocker of the N-type voltage dependent Ca^{2+} channels inhibited EFS-evoked tritium outflow, whereas it did not affect significantly basal (0.33 ± 0.02, $n=6$, $P>0.05$) and ATP-induced (Figure 2) tritium outflow. Similarly, Cd^{2+} (100 μM) which is known to inhibit all types of voltage dependent Ca^{2+} channels at this concentration (Miller, 1987) almost totally inhibited EFS-evoked outflow, while it did not decrease basal (0.31 ± 0.03, $n=6$, $P>0.05$) and ATP-evoked outflow, moreover, the latter was potentiated (Figure 2). Blockade of sodium-dependent action potential propagation by TTX (1 μM), which prevented electrical field stimulation-evoked [^{3}H]-NA outflow, did not significantly affect ATP-induced [^{3}H]-NA outflow (Figure 2).

Since a relatively high concentration of ATP was necessary to obtain this effect, the possibility that ATP might have been broken down rapidly in the extracellular space was envisaged in the following experiments. The decomposition of exogenous ATP was determined by HPLC-UV technique (Figure 3). Sixty nmol ATP, added to the preparations was readily hydrolyzed to ADP, AMP and adenosine by the ectoATPase, ectoATPdiphosphohydrolase and 5′nucleotidase enzymes, and as an end-product of inactivation, inosine also appeared in the extracellular fluid, showing the activity of the adenosine deaminase enzyme. The v_{max} and K_M values of ectoATPase enzyme, obtained in initial rate measurements were 14 ± 4.18 nmol min^{-1} prep. and 445 ± 92 μM respectively ($n=3$). Hence, ATP itself, but its breakdown products acting on adenosine receptors might be also responsible for increased outflow of tritium in response to ATP challenge. In the presence of DPCPX (250 nM), the A_1-selective adenosine receptor antagonist, and DMPX (250 nM), the relatively selective A_2 receptor antagonist the basal efflux of [^{3}H]-NA did not change significantly (0.49 ± 0.05, $n=6$, $P>0.05$ and 0.41 ± 0.02, $n=6$, $P>0.05$ in the presence of DPCPX and DMPX, respectively). Neither DPCPX (250 nM), nor DMPX (250 nM) affected [^{3}H]-NA outflow induced by 1 mM ATP (Figure 4). As it has been demonstrated that noradrenaline release from sympathetic neurons is under the control of inhibitory P_{2Y}-like purinoceptors (von Kugelgen *et al*., 1993; 1995) the effect of reactive blue 2 (RB2, 10 μM), a P_2 receptor antagonist known to antagonize this inhibitory neuromodulation was also tested: nevertheless, RB2 was ineffective to modify either basal (0.49 ± 0.24, $n=6$, $P>0.05$), or ATP (1 mM)-induced outflow (Figure 4). In contrast, PPADS (30 μM) another P_2 receptor-antagonist completely inhibited, while Zn^{2+} (50 μM) the allosteric modulator of ATP gated ion channels significantly potentiated ATP-induced response (Figure 4), while no change in basal efflux of tritium was observed (0.39 ± 0.03, $n=6$, $P>0.05$ and 0.31 ± 0.01, $n=8$, $P>0.05$, in the presence of PPADS and Zn^{2+}, respectively).

Apart from ATP, other agonists, known to act on P_2 receptors were also active to promote tritium release. Figure 5 shows the concentration-response curves of different P_2 agonists to elicit [^{3}H]-NA outflow. ATP appeared to be the most potent agonist, whereas 2-methyl-thioATP and α,β-methylene-ATP also behaved as less potent, but full agonists (Figure 5). ADP exhibited similar potency than α,β-methylene-ATP, however its effect declined at 1 mM and it released significantly less tritium than ATP at this concentration ($0.23 \pm 0.01\%$ of tissue tritium, $n=6$, $P<0.05$), indicating that it acts as a partial agonist. β,γ-methylene-L-ATP, a relatively selective antagonist of P_{2X1} receptors (Trezise *et al*.,

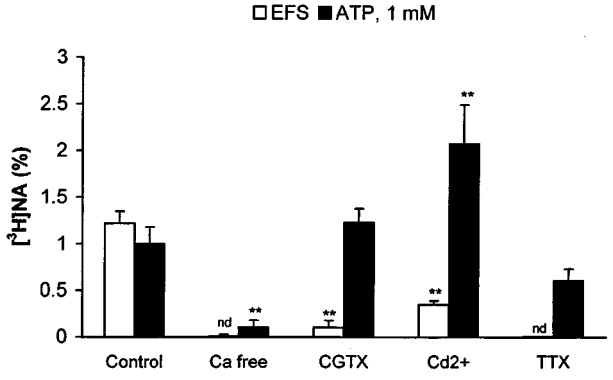

Figure 2 Effect of Ca^{2+} free medium, voltage dependent Ca^{2+} channel antagonists and tetrodotoxin on the electrical field stimulation-evoked and ATP-induced outflow of [^{3}H]-NA in the isolated guinea-pig right atrium. The preparations were superfused and subjected to electrical field stimulation (S_1, 25 V cm^{-1}, 2 Hz, 2.5 ms, 240 shocks) or ATP (1 mM) application, according to the experimental protocol shown in Figure 1b. Ca^{2+} free solution (Ca^{2+} free), supplemented with 1 mM EGTA was applied from 60 min before the start of sample collection, and thereafter, ω-conotoxin GVIA (CGTX, 0.1 μM), Cd^{2+} (Cd^{2+}, 100 μM) and tetrodotoxin (TTX, 1 μM) was perfused from 10 min before the start of the sample collection period, and thereafter. The net tritium release evoked by electrical field stimulation (EFS, open bars) or ATP (ATP, solid bars) were calculated by the area-under-the-curve method and expressed in fractional release (%). Data show the means±s.e.mean of 6–8 identical experiments. Asterisks indicate significant differences from respective controls, calculated by ANOVA followed by Dunnett test (**$P>0.01$). n.d. not detectable.

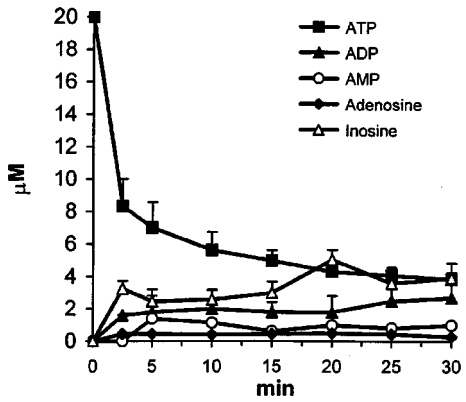

Figure 3 Extracellular decomposition of ATP in the isolated guinea-pig right atrium. Preparations were incubated in 3 ml Krebs' solution in the presence of 60 nmol ATP. Aliquots (70 μl) were taken 2.5, 5, 10, 20, 25, and 30 min after the addition of ATP. The amount of nucleotides (ATP, ADP, AMP) and nucleosides (ADO, adenosine, INO, inosine) in the aliquots were determined by HPLC-UV method and expressed in μM. Data show the means±s.e.mean of three identical experiments.

1995) appeared as an even weaker agonist, being ineffective at releasing substantial amounts of tritrium under our experimental conditions (Figure 5).

The effect of ATP was also tested in the presence of a variety of P_2 receptor antagonists. PPADS (30 μM), suramin (300 μM) and TNP-ATP (30 μM) all decreased or abolished the response obtained by ATP at different concentrations (Figure 6). NF279 (2 μM), an antagonist of P_{2X1} receptor did not affect significantly [^{3}H]-NA outflow evoked by 1 mM ATP (1.85 ± 0.22, $n=6$, $P>0.05$).

Since the above findings outlined a pharmacological profile resembling to P_{2X3} and P_{2X2}/P_{2X3} receptor, a RT–PCR study was performed to confirm that P_{2X2} and P_{2X3} receptor mRNA could be coexpressed in guinea-pig superior cervical ganglion (SCG) which is known to supply the sympathetic innervation of the heart.

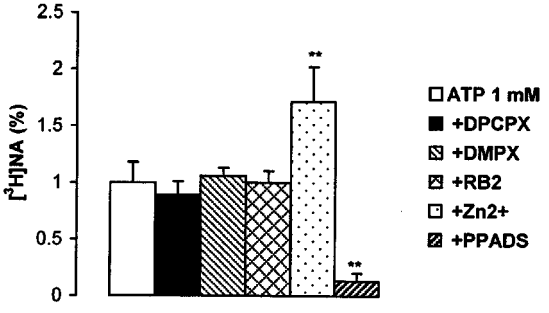

Figure 4 Effect of different antagonists and Zn^{2+} on ATP-induced outflow of [^{3}H]-NA. Isolated guinea-pig right atrium preparations were superfused and subjected to ATP (1 mM) application, according to the experimental protocol shown in Figure 1b. DPCPX (250 nM), DMPX (250 nM), reactive blue 2 (RB2, 10 μM), Zn^{2+} (50 μM) and PPADS (30 μM) were perfused 10 min before ATP application and thereafter. The net tritium release evoked by ATP was calculated by the area-under-the-curve method and expressed in fractional release (%). Data show the means ± s.e.mean of 6–8 identical experiments. $^{**}P<0.01$ indicates significant differences between ATP-evoked tritium outflow in the absence and presence of drugs or Zn^{2+}, calculated by ANOVA followed by Dunnett test.

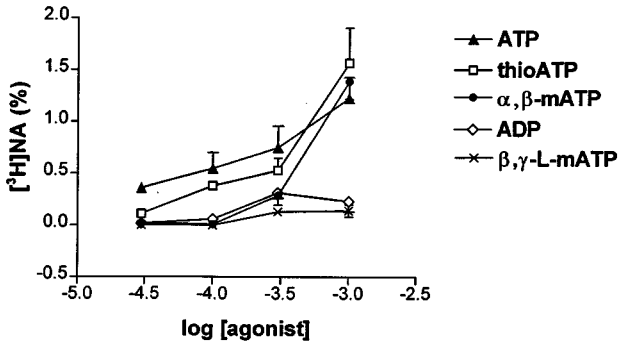

Figure 5 Concentration-response relationship of [^{3}H]-NA outflow evoked by different P_{2X} receptor agonists. Isolated guinea-pig right atrium preparations were superfused and subjected to ATP (triangles), ADP (diamonds), 2-methyl-thioATP (thioATP, squares), α,β-methylene-ATP (α,β-mATP, circles) or β,γ-methylene-L-ATP (β,γ-L-mATP, crosses) application in different concentrations indicated on the abscissa, ranging from 30 μM to 1 mM according to the experimental protocol shown in Figure 1b. The net tritium release evoked by different agonists were calculated by the area-under-the-curve method and expressed in fractional release (%). Data show the means ± s.e.mean of 6–8 identical experiments.

RT–PCR study

Specific primers were designed to the common regions of the known three splice variants of guinea-pig P_{2X2} receptor cDNA. Since the sequence of the guinea-pig P_{2X3} receptor cDNA has not been reported so far, in case of P_{2X3} receptor, primers designed to the rat P_{2X3} receptor cDNA were used. As shown in Figure 7, mRNAs encoding both P_{2X2} and P_{2X3} receptors were present in the guinea-pig SCG and hippocampus, the latter was used as a positive control. The length of both P_{2X2} and P_{2X3} specific PCR products were as expected. When PCR was performed on RNA samples without reverse transcription, there were no amplification products, indicating that the bands on the gel generated by RT–PCR couldn't be derived from direct amplification of genomic DNA.

Regulation of EFS-induced [^{3}H]-NA outflow by P_2 receptors activated by endogenous ATP

In order to exclude interaction of endogenous ATP with inhibitory modulation *via* A_1 and P_{2Y}-like receptors, the following experiments were carried out in the presence of

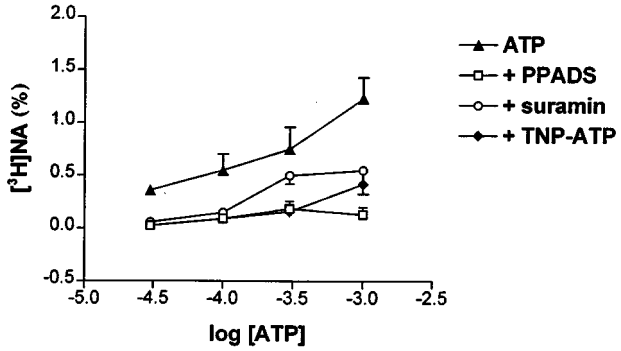

Figure 6 Effect of P_{2X} receptor antagonists on [^{3}H]-NA outflow evoked by ATP. Concentration-response curves for ATP were made in the absence (triangles) or presence of PPADS (30 μM, squares), suramin (300 μM, circles) and TNP-ATP (30 μM, diamonds). The net tritium release evoked by different concentrations of ATP ranging from 30 μM to 1 mM were calculated by the area-under-the-curve method and expressed in fractional release (%). Data show the means ± s.e.mean of 4–8 identical experiments.

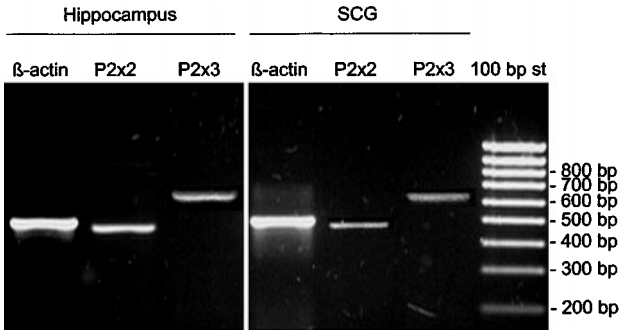

Figure 7 RT–PCR analysis of P_{2X2} and P_{2X3} receptor subtype expression in the guinea-pig SCG and hippocampus. Total RNA samples from the SCG and hippocampus were reverse transcribed and amplified by PCR using primers specific to P_{2X2} and P_{2X3} transcripts. Amplification of β-actin was used as internal control. A 100 bp DNA ladder (Fermentas, Vilnius, Lithuania) was used to identify PCR fragment sizes. The gels shown are representative of at least three independent experiments.

DPCPX (250 nM) and RB2 (10 μM), the antagonists of these receptors, which were perfused from the beginning of the sample collection period. Under these conditions the S_2/S_1 ratio was 1.14 ± 0.05 ($n=6$). When PPADS (30 μM) was perfused before the second stimulation, the resting outflow did not change significantly (data not shown). In contrast a slight but significant decrease in the EFS-evoked [^{3}H]-NA outflow was observed in the presence of PPADS: the S_2/S_1 ratio changed to 0.87 ± 0.06 ($n=6$, $P<0.01$).

Discussion

ATP has been known for a long time to act as an extracellular messenger in the cardiovascular system (Drury & Szent-Györgyi, 1929). It is co-released with noradrenaline from sympathetic nerve terminal in response to neuronal activity (Fredholm *et al*., 1982; Sperlágh & Vizi, 2000), but it could also be released from postsynaptic target cells (Vizi & Sperlágh, 1999; Vizi *et al*., 1992), i.e. from endothelial cells (Milner *et al*., 1990; Yang *et al*., 1994), and from cardiac muscle cells upon energy deprivation (Borst & Schrader, 1991). ATP activates multiple subtypes of ionotropic (P_{2X}) and metabotropic (P_{2Y}) receptors, regulating myocardial function, haemostasis and proliferation of the endothelium (Boarder & Hourani, 1998; Rongen *et al*., 1997). A further important site of action of extracellular ATP could be neuronal P_2 receptors, whereby neuroregulation of cardiac function could be locally and effectively controlled. Neuronal P_{2X} receptor subtypes have been extensively characterized in cellular systems (North & Surprenant, 2000; Ralevic & Burnstock, 1998), however much less is known about their identity and functional properties in whole tissues; in particular how they control neurotransmitter release. Here we provide pharmacological evidence that the release of NA is under the control of P_{2X} receptors, located on cardiac sympathetic nerve terminals.

In our experiments ATP and other P_2 receptor agonists elicited concentration-dependent [^{3}H]-NA outflow, which was comparable to that obtained after axonal stimulation of sympathetic nerves. ATP-induced [^{3}H]-NA outflow proved to be entirely dependent on extracellular Ca^{2+}, but apart from EFS-evoked outflow it was not abolished in the presence of voltage-dependent calcium channel blockers. Knowing that P_{2X} receptors have relatively high Ca^{2+} permeability (Rogers *et al*., 1997), the potential mechanism whereby ATP promotes the release of NA could be the direct influx of Ca^{2+} through the receptor ion channel complex, rather than the subsequent activation of voltage dependent calcium channels. On the other hand, blockade of voltage dependent sodium channels by TTX did not significantly affect ATP induced [^{3}H]-NA outflow, indicating that sodium channels do not play any part in the initiation of this kind of release. This result also suggests that the underlying receptor is located downstream from action potential initiation site and is supposedly at the varicose axon terminals of sympathetic neurons.

The concentration range of ATP to elicit this response was between 30 μM and 1 mM, which is relatively higher than values obtained in cellular systems where ectoATPase activity is inhibited or tightly controlled (Crack *et al*., 1995; Khakh & Kennedy, 1998). The most plausible explanation of this discrepancy is that ATP has broken down in the extracellular space to inactive metabolites by the ectonucleotidase cascade located on endothelial surface (Pearson *et al*., 1980). Therefore it was important to determine the degradation rate of ATP in this tissue. Indeed, exogenous ATP has been readily hydrolyzed to ADP, AMP and adenosine, due to the activity of ectoATPase, ectoATPdiphosphohydrolase and 5′nucleotidase enzymes, and as an end product of this inactivation pathway, inosine also appeared in the extracellular fluid due to the activity of adenosine deaminase enzyme. Nevertheless, neither DPCPX, the A_1-selective adenosine receptor antagonist, nor DMPX, the relatively selective A_2 receptor antagonist, affected significantly [^{3}H]-NA outflow induced by 1 mM ATP, suggesting that metabolites of ATP acting on adenosine receptors, did not contribute to this effect.

As several studies have shown, the release of NA from sympathetic nerve terminals is under the control of inhibitory P_{2Y}-like receptors (von Kugelgen *et al*., 1993; 1995), and this inhibitory neuromodulation is sensitive to RB2, an antagonist of P_2 receptors, the effect of this drug was also tested against ATP induced [^{3}H]-NA outflow. However, RB2 was ineffective to modify ATP-evoked [^{3}H]-NA outflow, showing that distinct receptors are responsible for inhibition and stimulation of NA release by extracellular ATP. In contrast other P_2 receptor antagonists, such as PPADS and suramin, inhibited, whereas Zn^{2+}, the allosteric modulator of certain ATP gated ion channels, potentiated ATP-induced [^{3}H]-NA outflow consistently with the idea that it is mediated by P_{2X} receptors. Although at present there is no ligand which is able to differentiate between P_{2X} and P_{2Y} receptor families, the notion, we favour, that P_{2X} rather than P_{2Y} receptors are responsible for this effect, relies on the following arguments: (1) The response was sensitive to modulation by Zn^{2+} and Cd^{2+}, and (2) was entirely $[Ca^{2+}]_o$-dependent, features characteristic for ATP gated ion channels (Li *et al*., 1993; Nakazawa & Ohno, 1997) but not to metabotropic P_{2Y} receptors; (3) ADP exhibited a weak, partial agonist effect which excluded the involvement of P_{2Y4} and P_{2Y6} receptors, where ADP is equipotent or more potent than ATP, whereas the agonist activity of α,β-methylene-ATP ruled out the involvement of P_{2Y1} and P_{2Y2} (Ralevic & Burnstock, 1998); (4) to date almost all P_{2Y}-like receptors which have been involved in the modulation of neurotransmitter release have been proved to be inhibitory receptor (e.g. von Kugelgen *et al*., 1993; 1995), while positive modulation of neurotransmitter release is generally mediated by P_{2X}-like receptors (e.g. Boehm, 1999; Gu & MacDermott, 1997; Hugel & Schlichter, 2000).

Our findings support previous observation of Boehm (1999) who found that cultured rat sympathetic neurons are equipped with presynaptic P_{2X} receptors, and confirm that these receptors are functional in a whole tissue, where synaptic organization and receptor distribution is retained. However, the pharmacological profile of the receptor identified in our experiments is dissimilar from that described in the above study, indicating that a different subtype of P_{2X} receptor is involved. Furthermore, the pharmacological pattern outlined by this study allows identification of the P_{2X} receptor subunit composition of the underlying receptor, at least from the known pharmacological phenotypes of P_{2X} receptor assemblies: these are six functional homomeric (P_{2X1}, P_{2X2}, P_{2X3}, P_{2X4}, P_{2X5}, P_{2X7}) and four heteromeric P_{2X}, (P_{2X2}/P_{2X3}, P_{2X1}/P_{2X5}, P_{2X2}/P_{2X6}, P_{2X4}/P_{2X6}) combinations (Haines *et al*., 1999; King *et al*., 2000; Le *et al*., 1998; 1999; North & Surprenant, 2000). As the most important feature, α,β-methylene-ATP acted as full agonist in our study, which rules out the involvement of all homomeric combinations, except P_{2X1} and P_{2X3}, and the heteromeric assemblies, except P_{2X2}/P_{2X3} and P_{2X4}/P_{2X6}. This finding is also indicative for the phenotypic difference between P_{2X} receptors regulating NA release in the rat and guinea-pig sympathetic

nerves, i.e. at the rat receptor which was identified as a P_{2X2} receptor, α,β-methylene-ATP was inactive (Boehm, 1999). Since β,γ-methylene-L-ATP, which has been claimed as a selective P_{2X1} receptor agonist (Trezise *et al*., 1995) was virtually inactive in our experiments, the involvement of P_{2X1} receptors seems also unlikely. Furthermore, reactive blue 2, which has been shown to antagonize responses mediated by P_{2X1} (Brake *et al*., 1994) and heterologously expressed P_{2X4}/P_{2X6} receptors (Le *et al*., 1998), did not affect the response evoked by ATP in our study, which limits the likely candidates to P_{2X3} or P_{2X2}/P_{2X3} combinations. Supporting this assumption, TNP-ATP that is reported to be active at these two (but not at all) P_{2X} receptors (Virginio *et al*., 1998) antagonized the response evoked by ATP. Although TNP-ATP has been reported as nanomolar affinity ligand at these receptor assemblies (Lewis *et al*., 1998; Virginio *et al*., 1998) it exhibited lower affinity in whole tissues, most likely because it is subject to dephosphorylation by ectonucleotidases (Lewis *et al*., 1998). The most important phenotypic difference between P_{2X3} and $P_{2X2/3}$ receptor is their different desensitization properties (Lewis *et al*., 1995); however, the temporal resolution of the release technique does not allow direct investigation of receptor desensitization. Instead, a RT–PCR study was carried out to prove whether these P_{2X} receptor subunits could be coexpressed in guinea-pig SCG, the sympathetic ganglion providing the sympathetic innervation of the cardiac tissue. In the case of P_{2X2} receptors, specific primers were designed to those regions of guinea-pig P_{2X2} cDNA which are present in all splice variants of this receptor subtype (Parker *et al*., 1998), and our data showed that this receptor is expressed in the hippocampus and in the SCG. We have used rat-specific P_{2X3} primers to detect the P_{2X3} receptor mRNA in a guinea-pig, since to date P_{2X2} receptor is the only P_{2X} subtype which has been molecularly identified in this species (Parker *et al*., 1998). Nevertheless mRNA for this receptor was also clearly detected both in the hippocampus and atrium, indicating that the rat primers could amplify the guinea-pig P_{2X3} receptor cDNA, and there should be high sequence homology between rat and guinea-pig P_{2X3} genes. This is not surprising, taking into account the relatively high homology between rat and guinea-pig P_{2X2} (Parker *et al*., 1998). Our data are also in agreement with the very recent immunohistochemical study of Zhong *et al*., who found that P_{2X2} and P_{2X3} receptors are the only P_{2X} subtypes which are expressed in guinea-pig SCG (Zhong *et al*., 2000a) as opposed to rat SCG, where P_{2X1}, P_{2X2}, P_{2X4} and P_{2X6} are expressed (Xiang *et al*., 1998). Therefore our data adds further support to the growing notion that the composition of P_{2X} receptor subunits expressed in sympathetic ganglia of rat and guinea-pig is different (Zhong *et al*., 2000a,b), and this species heterogeneity is also manifested at those receptors which are expressed at the nerve terminal level. Nevertheless, our data does not rule out the possibility that the expression pattern of P_{2X} receptors in cardiac sympathetic nerve terminals is inhomogeneous and individual nerve terminals might express different subunit assemblies i.e. either P_{2X3} or $P_{2X2/3}$ or even P_{2X2}.

As it is well known that endogenous ATP is co-released with NA from the sympathetic nerves (Sperlágh & Vizi, 2000; Vizi *et al*., 1992) and from the postsynaptic target cell upon neuronal stimulation (Vizi & Sperlágh, 1999; Vizi *et al*., 1992), and early studies showed stimulation-dependent release of ATP from sympathetic nerves supplying the heart (Fredholm *et al*., 1982), it seemed worthwhile to challenge the hypothesis, whether the release of noradrenaline evoked by axonal stimulation is subject to modulation by endogenous ATP through presynaptic P_{2X} receptors described in the previous experiments. Since NA release from sympathetic nerves is under the tonic control of inhibitory P_2 receptors (von Kugelgen *et al*., 1995) and A_1 adenosine receptors (Hedqvist & Fredholm, 1979), the possible interference of endogenous ATP with these receptors was prevented by the application of RB2 and DPCPX. Under these conditions PPADS (the P_2 receptor antagonist) slightly but significantly reduced the electrical field stimulation-induced NA outflow, raising the possibility that the release of NA upon neuronal activity is under the autofacilitatory control of P_2 receptors located on varicose terminals of sympathetic nerves. However, as PPADS has also inhibitory activity on ectoATPase enyzme (Bultmann *et al*., 1996) and on intracellular Ca^{2+} mobilization (Vigne *et al*., 1996) this assumption needs further investigation.

In summary, we show here that the release of NA from cardiac sympatheic nerves is regulated by presynaptic P_{2X} receptors. The pharmacological phenotype of this receptor is similar to homo-oligomeric P_{2X3} and to hetero-oligomeric P_{2X2}/P_{2X3} receptors, consistent with the expression of the receptor subunit mRNA in the SCG. These receptors may serve as a target site for endogenous ATP released from various sources (Sperlágh & Vizi, 2000) or drugs acting in a non-synaptic fashion (Vizi, 2000) in the cardiovascular system under physiological and pathological conditions.

This study was supported by grants from the Hungarian Research Foundation (OTKA T025614, T029859) and Hungarian Medical Research Council (ETT27/2000). The authors are grateful to Dr Joseph Simon for providing RT–PCR primers, for Ms Zsuzzsanna Körössy for excellent technical assistance, and for Ms Éva Szénássy for HPLC measurements.

References

AUSUBEL, F.M., BRENT, R., KINGTON, R.E., MOORE, D.D., SEIDMAN, J.G., SMITH, J.A., STRUHL, K. & CHANDA, V.B. (eds). (1988). Removal of contaminating DNA. In *Current Protocols in Molecular Biology*. Ch. 4.1.4. New York: John Wiley & Sons.

BOARDER, M.R. & HOURANI, S.M. (1988). The regulation of vascular function by P2 receptors: multiple sites and multiple receptors. *Trends Pharmacol. Sci.,* **19,** 99–107.

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

BORST, M.M. & SCHRADER, J. (1991). Adenine nucleotide release from isolated perfused guinea-pig hearts and extracellular formation of adenosine. *Circ. Res.,* **68,** 797–806.

BRAKE, A.J., WAGENBACH, M.J. & JULIUS, D. (1994). New structural motif for ligand-gated ion channels defined by an ionotropic ATP receptor. *Nature,* **371,** 519–523.

BULTMANN, R., PAUSE, B., WITTENBURG, H., KURZ, G. & STARKE, K. (1996). P2-purinoceptor antagonists: I. Blockade of P2-purinoceptor subtypes and ecto-nucleotidases by small aromatic isothiocyanato-sulphonates. *Naunyn Schmiedeberg's Arch. Pharmacol.,* **354,** 481–490.

CRACK, B.E., POLLARD, C.E., BEUKERS, M.W., ROBERTS, S.M., HUNT, S.F., INGALL, A.H., MCKECHNIE, K.C., AP, I.J. & LEFF, P. (1995). Pharmacological and biochemical analysis of FPL 67156, a novel, selective inhibitor of ecto-ATPase. *Br. J. Pharmacol.,* **114,** 475–481.

DRURY, A.N. & SZENT-GYÖRGYI, A. (1929). The physiological action of adenine compounds with especial reference to their action on the mammalian heart. *J. Physiol. (Lond).* **68,** 214–237.

EDWARDS, F.A., GIBB, A.J. & COLQUHOUN, D. (1992). ATP receptor-mediated synaptic currents in the central nervous system. *Nature,* **359,** 144 – 147.

EVANS, R.J., DERKACH, V. & SURPRENANT, A. (1992). ATP mediates fast synaptic transmission in mammalian neurons. *Nature,* **357,** 503 – 505.

EVANS, R.J. & SURPRENANT A. (1996). P2x receptors in autonomic and sensory neurons. *Semin. Neurosci.,* **8,** 217 – 223.

FREDHOLM, B.B., HEDQVIST, P., LINDSTROM, K. & WENNMALM, M. (1982). Release of nucleosides and nucleotides from the rabbit heart by sympathetic nerve stimulation. *Acta Physiol. Scand.,* **116,** 285 – 295.

GU, J.G. & MACDERMOTT, A.B. (1997). Activation of ATP P2X receptors elicits glutamate release from sensory neuron synapses. *Nature,* **389,** 749 – 753.

HAINES, W.R., TORRES, G.E., VOIGT, M.M. & EGAN, T.M. (1999). Properties of the novel ATP-gated ionotropic receptor composed of the P2X (1) and P2X(5) isoforms. *Mol. Pharmacol.,* **56,** 720 – 727.

HEDQVIST, P. & FREDHOLM, B.B. (1979). Inhibitory effect of adenosine on adrenergic neuroeffector transmission in the rabbit heart. *Acta Physiol. Scand.,* **105,** 120 – 122.

HUGEL, S. & SCHLICHTER R. (2000). Presynaptic P2X receptors facilitate inhibitory GABAergic transmission between cultured rat spinal cord dorsal horn neurons. *J. Neurosci.,* **20,** 2121 – 2130.

KHAKH, B.S. & KENNEDY, C. (1998). Adenosine and ATP: progress in their receptors' structures and functions. *Trends Pharmacol. Sci.,* **19,** 39 – 41.

KING, B.F., TOWNSEND-NICHOLSON, A., WILDMAN, S.S., THOMAS, T., SPYER, K.M. & BURNSTOCK, G. (2000). Coexpression of Rat P2X2 and P2X6 Subunits in Xenopus Oocytes. *J. Neurosci.,* **20,** 4871 – 4877.

LE, K.T., BABINSKI, K. & SEGUELA, P. (1998). Central P2X4 and P2X6 channel subunits coassemble into a novel heteromeric ATP receptor. *J. Neurosci.,* **18,** 7152 – 7159.

LE, K.T., BOUE-GRABOT, E., ARCHAMBAULT, V. & SEGUELA, P. (1999). Functional and biochemical evidence for heteromeric ATP-gated channels composed of P2X1 and P2X5 subunits. *J. Biol. Chem.,* **274,** 15415 – 15419.

LEWIS, C., NEIDHART, S., HOLY, C., NORTH, R.A., BUELL, G. & SURPRENANT, A. (1995). Coexpression of P2X2 and P2X3 receptor subunits can account for ATP-gated currents in sensory neurons. *Nature,* **377,** 432 – 435.

LEWIS, C.J., SURPRENANT, A. & EVANS, R.J. (1998). 2′,3′-O-(2,4,6-trinitrophenyl) adenosine 5′-triphosphate (TNP-ATP) – a nanomolar affinity antagonist at rat mesenteric artery P2X receptor ion channels. *Br. J. Pharmacol.,* **124,** 1463 – 1466.

LI, C., PEOPLES, R.W., LI, Z. & WEIGHT, F.F. (1993). Zn^{2+} potentiates excitatory action of ATP on mammalian neurons. *Proc. Natl. Acad. Sci. U.S.A.,* **90,** 8264 – 8267.

MACDERMOTT, A.B., ROLE, L.W. & SIEGELBAUM, S.A. (1999). Presynaptic ionotropic receptors and the control of transmitter release. *Annu. Rev. Neurosci.,* **22,** 443 – 485.

MILLER, R.J. (1987). Multiple calcium channels and neuronal function. *Science,* **235,** 46 – 52.

MILNER, P., BODIN, P., LOESCH, A. & BURNSTOCK, G. (1990). Rapid release of endothelin and ATP from isolated aortic endothelial cells exposed to increased flow. *Biochem. Biophys. Res. Commun.,* **170,** 649 – 656.

NAKATSUKA, H., NAGANO, O., FOLDES, F.F., NAGASHIMA, H. & VIZI, E.S. (1995). Effects of adenosine on norepinephrine and acetylcholine release from guinea-pig right atrium: role of A1 receptors. *Neurochem. Int.,* **27,** 345 – 353.

NAKAZAWA, K. & OHNO. Y. (1997). Effects of neuroamines and divalent cations on cloned and mutated ATP-gated channels. *Eur. J. Pharmacol.,* **325,** 101 – 108.

NORTH, R.A. & SURPRENANT, A. (2000). Pharmacology of cloned P2X receptors. *Annu. Rev. Pharmacol. Toxicol.,* **40,** 563 – 580.

OE, K., SPERLAGH, B., SANTHA, E., MATKO, I., NAGASHIMA, H., FOLDES, F.F. & VIZI, E.S. (1999). Modulation of norepinephrine release by ATP-dependent $K(^{+})$-channel activators and inhibitors in guinea-pig and human isolated right atrium. *Cardiovasc Res.,* **43,** 125 – 134.

PARKER, M.S., LARROQUE M.L., CAMPBELL, J.M., BOBBIN, R.P. & DEININGER, P.L. (1998). Novel variant of the P2X2 ATP receptor from the guinea-pig organ of Corti. *Hear. Res.,* **121,** 62 – 70.

PEARSON, J.D., CARLETON, J.S. & GORDON, J.L. (1980). Metabolism of adenine nucleotides by ectoenzymes of vascular endothelial and smooth-muscle cells in culture. *Biochem. J.,* **190,** 421 – 429.

RALEVIC, V. & BURNSTOCK, G. (1998). Receptors for purines and pyrimidines. *Pharmacol. Rev.,* **50,** 413 – 492.

ROGERS, M., COLQUHOUN, L.M., PATRICK, J.W. & DANI, J.A. (1997). Calcium flux through predominantly independent purinergic ATP and nicotinic acetylcholine receptors. *J. Neurophysiol.,* **77,** 1407 – 1417.

RONGEN, G.A., FLORAS, J.S., LENDERS, J.W., THIEN, T. & SMITS, P. (1997). Cardiovascular pharmacology of purines. *Clin. Sci. (Colch),* **92,** 13 – 24.

SPERLAGH, B., KITTEL, A., LAJTHA, A. & VIZI, E.S. (1995). ATP acts as fast neurotransmitter in rat habenula: neurochemical and enzymecytochemical evidence. *Neurosci,* **66,** 915 – 920.

SPERLAGH, B. & VIZI, E.S. (1991). Effect of presynaptic P2 receptor stimulation on transmitter release. *J. Neurochem.,* **56,** 1466 – 1470.

SPERLAGH, B. & VIZI, E.S. (2000). Regulation of purine release. In *Handbook of Experimental Pharmacology. Purinergic and pyrimidinergic signalling*. (ed.) Abbracchio, M.P., Williams, M. Heidelberg. Springer: (in press).

SUN, X.P. & STANLEY, E.F. (1996). An ATP-activated, ligand-gated ion channel on a cholinergic presynaptic nerve terminal. *Proc. Natl. Acad. Sci. U.S.A.,* **93,** 1859 – 1863.

TOKUNAGA, K., TANIGUCHI, H., YODA, K., SHIMIZU, M. & SAKIYAMA, S. (1986). Nucleotide sequence of a full-length cDNA for mouse cytoskeletal beta-actin mRNA. *Nucleic Acids Res.,* **14,** 28 – 29.

TORRES, G.E., EGAN, T.M. & VOIGT, M.M. (1999). Hetero-oligomeric assembly of P2X receptor subunits. Specificities exist with regard to possible partners. *J. Biol. Chem.*. **274,** 6653 – 6659.

TREZISE, D.J., MICHEL, A.D., GRAHAMES, C.B., KHAKH, B.S., SURPRENANT, A. & HUMPHREY, P.P. (1995). The selective P2X purinoceptor agonist, beta, gamma-methylene-L-adenosine 5′-triphosphate, discriminates between smooth muscle and neuronal P2X purinoceptors. *Naunyn Schmiedeberg's Arch. Pharmacol.,* **351,** 603 – 609.

VIGNE, P., PACAUD, P., URBACH, V., FEOLDE, E., BREITTMAYER, J.P. & FRELIN, C. (1996). The effect of PPADS as an antagonist of inositol (1,4,5)trisphosphate induced intracellular calcium mobilization. *Br. J. Pharmacol.,* **119,** 360 – 364.

VIRGINIO, C., ROBERTSON, G., SURPRENANT, A. & NORTH, R.A. (1998). Trinitrophenyl-substituted nucleotides are potent antagonists selective for P2X1, P2X3, and heteromeric P2X2/3 receptors. *Mol. Pharmacol.,* **53,** 969 – 973.

VIZI, E.S. (2000). Role of high-affinity receptors and membrane transporters in non-synaptic communication and drug action in the central nervous system. *Pharmacol. Rev.,* **52,** 63 – 90.

VIZI, E.S. & SPERLAGH, B. (1999). Receptor- and carrier-mediated release of ATP of postsynaptic origin: cascade transmission. *Prog. Brain. Res.,* **120,** 159 – 169.

VIZI, E.S., SPERLAGH, B. & BARANYI, M. (1992). Evidence that ATP released from the postsynaptic site by noradrenaline, is involved in mechanical responses of guinea-pig vas deferens: cascade transmission. *Neurosci.,* **50,** 455 – 465.

VON KUGELGEN, I., KURZ, K. & STARKE, K. (1993). Axon terminal P2-purinoceptors in feedback control of sympathetic transmitter release. *Neurosci.,* **56,** 263 – 267.

VON KUGELGEN, I., NÖRENBERG, W., KOCH, A., MEYER, A., ILLES, P. & STARKE, K. (1999). P2 receptors controlling neurotransmitter release from postganglionic sympathetic neurones. *Prog. Brain. Res.,* **120,** 173 – 183.

VON KUGELGEN, I., STOFFEL, D. & STARKE, K. (1995). P2-purinoceptor-mediated inhibition of noradrenaline release in rat atria. *Br. J. Pharmacol.,* **115,** 247 – 254.

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

YANG, S., CHEEK, D.J., WESTFALL, D.P. & BUXTON, I.L. (1994). Purinergic axis in cardiac blood vessels. Agonist-mediated release of ATP from cardiac endothelial cells. *Circ. Res.,* **74,** 401 – 407.

ZHANG, Y.X., YAMASHITA, H., OHSHITA, T., SAWAMOTO, M. & NAKAMURA, S. (1996). ATP induces release of newly synthesized dopamine in the rat striatum. *Neurochem. Int.*, **28,** 395–400.

ZHONG, Y., DUNN, P.M. & BURNSTOCK, G. (2000a). Guinea-pig sympathetic neurons express varying proportions of two distinct P2X receptors. *J. Physiol. (Lond.),* **523,** 391–402.

ZHONG, Y., DUNN, P.M. & BURNSTOCK, G. (2000b). Pharmacological comparison of P2X receptors on rats coeliac, mouse coeliac and mouse pelvic ganglion neurons. *Neuropharmacol.,* **39,** 172–180.

(Received July 24, 2000
Revised September 28, 2000
Accepted October 6, 2000)