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ScienceDirect
Neuropharmacology 54 (2008) 375–386
NEURO PHARMACOLOGY
www.elsevier.com/locate/neuropharm

Modulation of neurotransmitter release by P2X and P2Y receptors in the rat spinal cord

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Received 16 July 2007; received in revised form 28 September 2007; accepted 15 October 2007

Abstract

In this study, the P2 receptor-mediated modulation of [^{3}H]glutamate and [^{3}H]noradrenaline release were examined in rat spinal cord slices. Adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP), and 2-methylthioadenosine 5′-diphosphate (2-MeSADP) decreased the electrical stimulation-evoked [^{3}H]glutamate efflux with the following order of potency: ADP > 2-MeSADP > ATP. The effect of ATP was antagonized by suramin (300 μM), the $P2Y_{12,13}$ receptor antagonist 2-methylthioadenosine 5′-monophosphate (2-MeSAMP, 10 μM), and partly by 4-[[4-Formyl-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-2-pyridinyl]azo]-1,3-benzenedisulfonic acid (PPADS, 30 μM) and the $P2Y_1$ receptor antagonist 2′-deoxy-N^6-methyladenosine 3′,5′-diphosphate (MRS 2179, 10 μM). ATP, ADP and 2-MeSADP also decreased evoked [^{3}H]noradrenaline outflow; the order of agonist potency was ADP ≥ 2-MeSADP > ATP. The effect of ATP was reversed by 2-MeSAMP (10 μM), and partly by MRS 2179 (10 μM). By contrast, 2-methylthioadenosine-5′-triphosphate (2-MeSATP, 10–300 μM) increased resting and electrically evoked [^{3}H]glutamate and [^{3}H]noradrenaline efflux, and this effect was prevented by the $P2X_1$ receptor selective antagonist 4,4′,4″,4‴-[carbonylbis[imino-5,1,3-benzenetriyl bis (carbonyl-imino)]] tetrakis (benzene-1,3-disulfonic acid) octasodium salt (NF449, 100 nM). Reverse transcriptase polymerase chain reaction (RT-PCR) analysis revealed that mRNAs encoding $P2Y_{12}$ and $P2Y_{13}$ receptors are expressed in the brainstem, whereas $P2Y_{13}$ but not $P2Y_{12}$ receptor mRNA is present in the dorsal root ganglion and spinal cord. $P2Y_1$ receptor expression in the spinal cord is also demonstrated at the protein level. In conclusion, inhibitory P2Y and facilitatory $P2X_1$-like receptors, involved in the regulation of glutamate ($P2Y_{13}$ and/or $P2Y_1$) and noradrenaline ($P2Y_{13}$ and/or $P2Y_1$, $P2Y_{12}$) release have been identified, which provide novel target sites for analgesics acting at the spinal cord level.

Keywords: ATP; Glutamate; Release; Noradrenaline; Spinal cord; P2 receptors

1. Introduction

Under normal conditions, pain is associated with electrical activity in small-diameter fibers of dorsal root ganglion (DRG) of the spinal cord. In addition, numerous studies have shown that descending pathways from the brainstem, including the descending noradrenergic pathway play crucial role in the modulation of sensory transmission in the spinal cord and thereby attenuate pain sensation (Millan, 2002).

The ability of ATP to elicit pain was first described more than 40 years ago (Collier et al., 1966), and it is now widely recognized that it is an important messenger involved in sensory information processing (Burnstock, 2006a). ATP is released from spinal cord nerve terminals upon depolarization (Sawynok et al., 1993) and probably from damaged or stressed cells upon pathological conditions (cf. Sperlágh, in press). The released ATP acts via various subtypes of ionotropic P2X (homomeric $P2X_{1-7}$, and hetero-oligomeric $P2X_{1/2}$, $P2X_{1/5}$, $P2X_{2/3}$, $P2X_{1/4}$, $P2X_{2/6}$, $P2X_{4/6}$ receptors, (Roberts et al., 2006) and/or metabotropic P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ receptors, Abbracchio et al., 2006). mRNA encoding all subunits of the P2X receptors are expressed along the nociceptive pathways, including the DRG

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doi:10.1016/j.neuropharm.2007.10.013

(Dunn et al., 2001; Ruan et al., 2005), and among them, $P2X_1$, $P2X_2$ and $P2X_3$ receptor proteins are expressed on different subpopulations of primary afferent neurons (Bradbury et al., 1998; Petruska et al., 2000). ATP, activating P2X receptors, acts as an excitatory neurotransmitter in the dorsal horn of the spinal cord (Bardoni et al., 1997). Moreover, the activation of P2X receptors not only mediates but also facilitates excitatory transmission, releasing glutamate from primary afferent fibers terminating in lamina II (Li and Perl, 1995; Gu and MacDermott, 1997; Nakatsuka and Gu, 2001; Nakatsuka et al., 2003) and lamina V (Nakatsuka et al., 2003) of the spinal cord; these actions are mediated by $P2X_3$, $P2X_{1/5}$ and $P2X_{4/6}$ receptors. Less is known about the role of metabotropic P2 receptors in the modulation of signal transmission in the spinal cord. All subtypes of the P2Y receptor family are widely expressed in different parts of the nervous system, although the expression of only $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptor has been demonstrated so far in sensory neurons (Hussl and Boehm, 2006). The activation of P2Y receptors causes blockade of the N-type calcium channels in DRG cells (Borvendeg et al., 2003). This effect may decrease the release of glutamate from DRG terminals in the spinal cord and thereby partly counterbalance the algogenic effect of ATP (Gerevich and Illes, 2004; Gerevich et al., 2004). Nevertheless, neurochemical evidence supporting a role for these receptors in the modulation of spinal neurotransmitter release has not been presented so far.

Therefore, in this study we: (1) examined the effect of P2 receptor activation on the release of [^{3}H]glutamate ([^{3}H]GLU) and [^{3}H]noradrenaline ([^{3}H]NA) from rat spinal cord slices; (2) explored the expression of different P2Y receptor subtypes in the rat brainstem, spinal cord and DRG at the mRNA and protein level; and (3) an attempt was made to identify the underlying receptor subunits.

2. Materials and methods

All studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals, and were approved by the local Animal Care Committee of the Institute of Experimental Medicine.

2.1. Tritium outflow experiments

[^{3}H]GLU and [^{3}H]NA release experiments were performed by the application of the method described in our previous studies in the spinal cord (Sperlágh et al., 2002; Papp et al., 2004). Briefly, male Wistar rats (140–160 g, bred in the local animal house) were anesthetized under light CO_2 inhalation, and then decapitated. The spinal cord was dissected in ice-cold Krebs solution saturated with 95% O_2 and 5% CO_2, and 400 μm thick slices were prepared using a McIlwain Tissue Chopper 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 7.4) containing 1 μCi/ml [^{3}H]GLU (specific activity 49 Ci/mmol) or 2.5 μCi/ml [^{3}H]NA (specific activity 33 Ci/mmol), ascorbic acid (300 μM) and Na_2EDTA (30 μM) for 30 min. The medium was bubbled continuously with 95% O_2 and 5% CO_2 and maintained at 37 °C ([^{3}H]NA); in order to minimize the spontaneous firing of excitatory neurons and the metabolic efflux of [^{3}H]GLU, the bath temperature was kept at 32 °C in [^{3}H]GLU release experiments. After incubation, the tissues were rinsed three times with 6 ml Krebs solution, transferred to polypropylene tissue chambers and superfused continuously with 95% O_2- and 5% CO_2-saturated modified Krebs solution at a rate of 0.65 ml/min. In order to wash out the excess radioactivity and to allow tissue equilibration, a 60-min preperfusion time was applied and subsequently, 3-min perfusate samples were collected and assayed for [^{3}H]GLU or [^{3}H]NA. The slices were electrically stimulated during the collection period using platinum ring electrodes (diameter: 5 mm) fixed to the top and the bottom of the 100 μL volume tissue chamber, with the following parameters: 40 V, 15 Hz, 3.5 ms, 1 min ([^{3}H]GLU) and 40 V, 3 Hz, 1 ms, 2 min ([^{3}H]NA). The radioactivity released from the preparations was measured using a Packard 1900 Tricarb liquid scintillation spectrometer (Packard, Canberra, Australia). A 0.5 ml aliquot of the perfusate sample was added to 2 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]neurotransmitters was expressed in Bq/g and as a percentage of the amount of radioactivity in the tissue at the sample collection time (fractional release). The tissue tritium uptake was determined as the sum of the release plus the tissue content after the experiment, and was expressed in Bq/g. Electrical stimulation-induced [^{3}H]NA and [^{3}H]GLU efflux (EFS_1, EFS_2) was expressed by calculating the net release in response to electrical stimulation by the area-under-the-curve method – that is, by subtracting the release before the electrical stimulation from the values measured after stimulation. P2 receptor agonists (ATP, adenosine 5′-diphosphate [ADP], 2-methylthioadenosine-5′-triphosphate [2-MeSATP], 2-methylthioadenosine 5′-diphosphate [2-MeSADP]) were perfused from 18 min before the second stimulation and thereafter. Their effects on the electrically evoked release of [^{3}H]NA and [^{3}H]GLU were expressed as EFS_2/EFS_1 ratios measured in the absence and presence of antagonists and other drugs (4-[[4-Formyl-5-hydroxy-6-methyl-3-[(phosphonooxy)methyl]-2-pyridinyl]azo]-1,3-benzenedisulfonic acid tetrasodium salt [PPADS], 4,4′,4″,4‴-[carbonylbis[imino-5,1,3-benzenetriyl bis (carbonyl-imino)]] tetrakis (benzene-1,3-disulfonic acid) octasodium salt [NF449], 2′-deoxy-N^6-methyladenosine 3′,5′-diphosphate diammonium salt [MRS 2179], 2-methylthioadenosine 5′-monophosphate [2-MeSAMP], suramin, 6-cyano-7-nitroquinoxaline-2,3-dione-disodium [CNQX], D(−)-2-amino-5-phosphonopentanoic acid [AP-5], bicuculline, 8-cyclopentyl-1,3-dipropylxanthine [DPCPX]), which were preperfused from 18 min before the first stimulation period until the end of the sample collection period. When Ca^{2+}-free solution was used, $CaCl_2$ was omitted from the Krebs′ solution and 1mM EGTA was added from the beginning of the preperfusion period. For the calculation of resting tritium efflux, the tritium content of the sample collected immediately before the second stimulation period was taken into account in the absence and presence of drugs, respectively.

Previous high-performance liquid chromatography (HPLC) analyses using similar protocols showed that majority of tritium efflux released by electrical field stimulation represents [^{3}H]NA and [^{3}H] excitatory amino acids. Therefore, tritium release was used as a marker endogenous release of transmitters; however, for the sake of simplicity, we refer to the efflux of [^{3}H] as [^{3}H]glutamate or [^{3}H]NA release.

2.2. RT-PCR amplification of different P2Y receptor mRNAs

Male Wistar rats (140–160 g) were decapitated under light CO_2 anesthesia and the brainstem, spinal cord, and DRG were quickly put into ice-cold Krebs solution oxygenated with 95% O_2 and 5% CO_2. Total RNA from the tissue samples was isolated with Trizol Isolation Reagent according to the protocol provided by the supplier (Invitrogen Life Technologies, Rockville, MD, USA). RNA (1 μg, 2 μl) was reverse transcribed using a RevertAid First Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Aliquots of the first-strand cDNA template were subjected to PCR using 0.4 μM (1 μl) forward and reverse primers and 2 U (0.3 μl) of Taq DNA Polymerase (Promega, Madison, WI, USA). The primers used for amplification of P2Y receptor cDNAs were: for $P2Y_{12}$ CAGGTTCTCTTCCCATTGCT (forward primer) and CAGCAATGATGATGAAAACC (reverse primer), for $P2Y_{13}$ GGCATCA ACCGTGAAGAAAT (forward primer) and GGGCAAAGCAGACAAAG AAG (reverse primer), for β-actin ATGGATGACGATATCGCTG (forward primer) and ATGAGGTAGTCTGTCAGGT (reverse primer). The GenBank

accession numbers were: $P2Y_{12}$: NM022800, $P2Y_{13}$: NM001002853, β-actin: X03765. The conditions for amplification were as follows: initial denaturation at 95 °C for 5 min, hot start at 80 °C, then 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 1 min, for 40 cycles, with a final extension at 72 °C for 5 min. PCR products were analyzed by agarose gel electrophoresis. The identity of the various amplified PCR products had previously been verified by sequencing. Genomic DNA contamination in RNA samples was ruled out by direct PCR amplification of RNA samples.

2.3. $P2Y_1$ receptor immunohistochemistry

Male Wistar rats (140–160 g) were decapitated and the spinal cord was quickly removed and placed into a fixative solution containing 4% paraformaldehyde (Merck, Darmstadt, Germany) in 0.1 M phosphate buffer (PB) at pH 7.4 for 30 min at room temperature. After several changes of the fixative and overnight fixation at 4 °C, fixative was washed out in 0.1 M PB (pH 7.4). Transverse cervical sections (35 μm) were cut by vibratome.

2.3.1. Immunofluorescence staining

Sections were incubated in blocking solution (5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS)) for 1 h. An incubation, with the first antibodies vesicular glutamate transporter 1 (VGLUT1) (1:3000, rabbit polyclonal, affinity-purified fusion protein containing amino acid residues 456–560 of rat VGLUT1, Synaptic Systems, Goettingen, Germany) or $P2Y_1$ (1:200, rabbit polyclonal corresponding to residues 242–258 of rat or human $P2Y_1$, Alomone Labs, Jerusalem, Israel), was performed at 4 °C overnight. After careful washing with PBS, incubation with the second antibodies (1:500 Alexa Fluor® 488 goat anti-rabbit IgG or Alexa Fluor® 594 goat anti-rabbit IgG, Molecular Probes, Invitrogen, Carlsbad, CA, USA) were carried out at room temperature for 2 h in the dark. After wash in distilled water, mounting in VectaShield (Vector Laboratories, Burlingame, CA, USA), pictures were taken by means of Nikon Eclipse E600 microscope (Nikon Corporation, Tokyo, Japan) equipped with a SPOT RT color digital camera (Diagnostic Instrument Inc. Sterling Heights, MI, USA). Captured images were printed using Adobe Photoshop 8.0 Software (Adobe Systems, Mounted View, CA, USA). Control experiments were performed using fresh blocking serum instead of the first antibody.

2.3.2. Immunohistochemical staining for bright field and electron microscopy

Endogenous peroxide activity were blocked by 3% H_2O_2 (15 min), traces of H_2O_2 were removed with 0.1 M PBS. Triton X100 (0.1%, 15 min) was applied to increase the penetration of the antibodies. Careful washing steps, incubation with blocking serum (5% normal goat serum for 2 h) then incubation with the first rabbit polyclonal antibodies, such as 1:3000 VGLUT1 (Synaptic Systems), or 1:200 $P2Y_1$ (Alomone Labs), were performed. After repeated washing, incubation with biotinylated anti-rabbit IgG for 2 h was carried out. An ABC-3,3 diaminobenzidine (DAB) staining kit was used according to the manufacturer's instructions (Vector Laboratories).

Sections for light microscopic investigation were washed and dried onto microscopic slides and mounted in Canada balsam. Pictures were taken under a Zeiss Axioplan2 microscope equipped with an Olympus 70D camera using DPC Controller software (Olympus Ltd., Tokyo, Japan).

Samples for electron microscopic investigation were washed and post-fixed in 1% OsO_4 (Taab Equipment Ltd., Aldermaston, Berkshire, England) for 30 min, dehydrated in graded ethanol (en bloc-stained with 2% uranyl acetate in 70% ethanol for 30 min) and embedded in Taab 812 resin. Ultrathin sections were cut and examined in a Hitachi 2001 transmission electron microscope (Hitachi, Tokyo, Japan). In control experiments the first antibody was omitted from the incubation medium.

2.4. Drugs

[^{3}H]NA and [^{3}H]GLU were obtained from Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, U.K. The other drugs used were: ADP, ATP, 2-MeSAMP, 2-MeSATP, MRS 2179, PPADS, DPCPX (all from Sigma-Aldrich Chemical Co., St. Louis, MO, USA), 2-MeSADP, NF449, CNQX, D-AP5, (−)-bicuculline methobromide (all from Tocris Bioscience, Ellisville, MO, USA), suramin (Germanin, Bayer AG, Leverkusen, Germany). All of the general reagents were purchased from Sigma-Aldrich Chemical Co., unless otherwise specified. All solutions were freshly prepared on the day of use.

2.5. Statistics

All data were expressed as means ± the standard error of the mean of n observations. The statistical analyses were made by one-way analysis of variance (ANOVA) followed by the Dunnett test (multiple comparisons) or Student's t-test (pair-wise comparisons). P values of less than 0.05 were considered statistically significant. IC_{50} value was calculated by fitting the data to sigmoidal logistic equations using the program Prism 3.01 (Graph Pad, San Diego, CA).

3. Results

3.1. [^{3}H]GLU release experiments

The overall radioactivity uptake of the slices and the basal and electrically evoked tritium overflow from the rat spinal cord in different experimental conditions are shown in Table 1. The basal neurotransmitter outflow measured in a 3-min sample in [^{3}H]GLU release experiments was 3.09 ± 0.16%, of the total tissue content ($n = 8$), which remained relatively constant during the subsequent sample collections. Electrical field stimulations (for parameters, see Section 2) were applied during the 3rd and 14th sample collections, resulting in a rapid

Table 1
Radioactivity uptake, electrically evoked tritium outflow (EFS_1, EFS_2), and the basal tritium outflow from the rat spinal cord

	Uptake (Bq/g)	Evoked outflow (%)		Basal outflow (%)
		EFS_1	EFS_2	
[^{3}H]GLU	$1.07 \pm 0.29 \times 10^5$ (8)	6.330 ± 0.342 (8)	6.016 ± 0.040 (8)	3.097 ± 0.160 (8)
[^{3}H]NA	$2.55 \pm 0.56 \times 10^5$ (8)	1.363 ± 0.136 (8)	1.136 ± 0.083 (8)	0.437 ± 0.015 (8)

Rat spinal cord slices were incubated with [^{3}H]glutamate and [^{3}H]noradrenaline for 30 min, then superfused with Krebs solution for 60 min; electrical field stimulation was then applied during the 3rd (EFS_1) and 14th (EFS_2) sample collection periods. Uptake of radioactivity was expressed in Bq/g (for calculation see Section 2), whereas outflow of [^{3}H]neurotransmitters was expressed as a percentage of the amount of radioactivity in the tissue at the time of sample collection (fractional release, %). Basal efflux of tritiated glutamate and noradrenaline was measured in a 3-min sample, collected immediately before the second stimulation period. Electrical stimulation-induced [^{3}H]glutamate and [^{3}H]noradrenaline efflux (EFS_1, EFS_2) was expressed by calculating the net release in response to electrical stimulation by the area-under-the-curve method – that is, by subtracting the release before the electrical stimulation from the values measured after stimulation. Values are means ± SEM for n control preparations. The number of experiments is in parentheses.

increase in the basal [^{3}H]GLU efflux, which peaked 3 min after EFS_1 and EFS_2, then gradually declined and returned to the baseline level (Fig. 1A). The amount of tritium released by the second stimulation period was comparable in amount to the first, resulting in an EFS_2/EFS_1 ratio of 1.01 ± 0.07 ($n = 8$) in control experiments. When the slices were superfused with Ca^{2+}-free Krebs′ solution supplemented with 1 mM EGTA, the evoked release of [^{3}H]GLU was inhibited more than 90%, without affecting the basal efflux (data not shown). The majority of the release could be therefore regarded as a Ca^{2+}-dependent release.

In subsequent experiments, the effect of different P2 receptor agonists on electrically evoked [^{3}H]GLU release was examined. Among them, ATP, ADP and 2-MeSADP all concentration-dependently attenuated the stimulation-evoked release of [^{3}H]GLU at the micromolar concentration range with the following rank order of agonist potency: ADP > 2-MeSADP > ATP (Figs. 1B and 2), whereas they did not affect basal tritium efflux (data not shown). The maximal inhibition of [^{3}H]GLU efflux obtained by ATP was $63.15 \pm 2.88\%$ ($n = 8$), and the apparent IC_{50} value was 70.1 μM. Among P2 receptor agonists, 2-MeSATP, the metabolically relatively stable analogue of ATP, at 100 μM concentration significantly inhibited, but in higher concentrations (200–300 μM) increased stimulation evoked [^{3}H]GLU overflow from the rat spinal cord slices (Figs. 1C and 2).

Next, the effect of ATP and 2-MeSATP on [^{3}H]GLU efflux was tested in the presence of various antagonists acting on P2 and P1 receptors (Fig. 3). The inhibitory effect of ATP (1 mM) on the evoked release of [^{3}H]GLU was abolished in the presence of the non-selective P2 receptor antagonist suramin (300 μM) and the $P2Y_{12,13}$ receptor selective antagonist 2-MeSAMP (10 μM); moreover, in the presence of 2-MeSAMP, a net potentiation was observed (Fig. 3A). By contrast, PPADS (30 μM) and MRS 2179, the selective $P2Y_1$ antagonist (10 μM) partly reversed the inhibitory effect of ATP ($38.96 \pm 8.1\%$, and $32.63 \pm 1.36\%$ inhibition in the presence of PPADS 30 μM and MRS 2179 10 μM, respectively, $n = 4–8$,

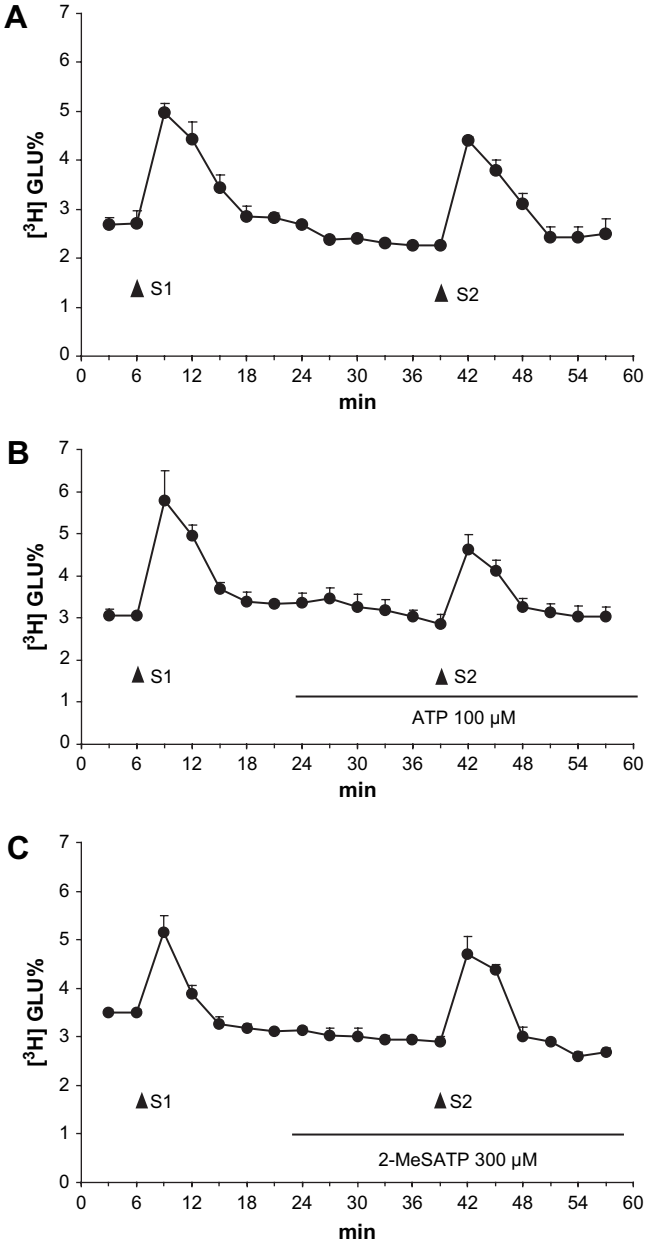

Fig. 1. Electrically induced [^{3}H]glutamate ([^{3}H]GLU) release from superfused rat spinal cord slices by nucleotides. Rat spinal cord slices were labeled with [^{3}H]GLU and superfused. Subsequent to a 60-min washout period, 3-min fractions of superfusate were collected. Preparations were stimulated twice (S_1, S_2; 40 V, 15 Hz, 3.5 ms, 1 min) in the absence (A) or presence of (B) adenosine 5′-triphosphate (ATP, 100 μM), (C) 2-MeSATP (300 μM), as indicated. The values show the mean ± SEM of 4–8 identical experiments.

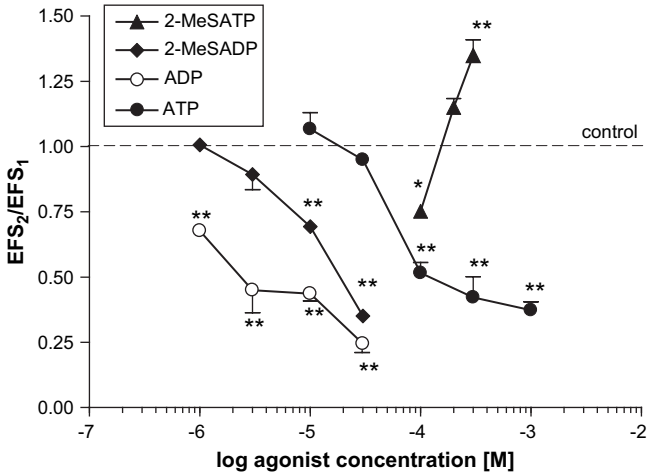

Fig. 2. Concentration-response curves of the effect of ATP (10 μM to 1 mM), adenosine 5′-diphosphate (ADP, 1 μM to 30 μM), 2-methylthioadenosine 5′-diphosphate (2-MeSADP, 1 μM to 30 μM) and 2-methylthioadenosine 5′-triphosphate (2-MeSATP, 100 μM to 300 μM) on [^{3}H]GLU release from rat spinal cord slices. The apparent order of potency of nucleotides that decreased evoked tritium overflow was ADP > 2-MeSADP > ATP. The results are expressed as EFS_2/EFS_1 ratios in the presence of various concentrations of agonists as indicated on the abscissa. A dotted line represents the control value. The values show the mean ± SEM of 4–8 identical experiments. Asterisks indicate significant differences from the respective controls, calculated by one-way analysis of variance, followed by the Dunnett test. (*$P < 0.05$; **$P < 0.01$).

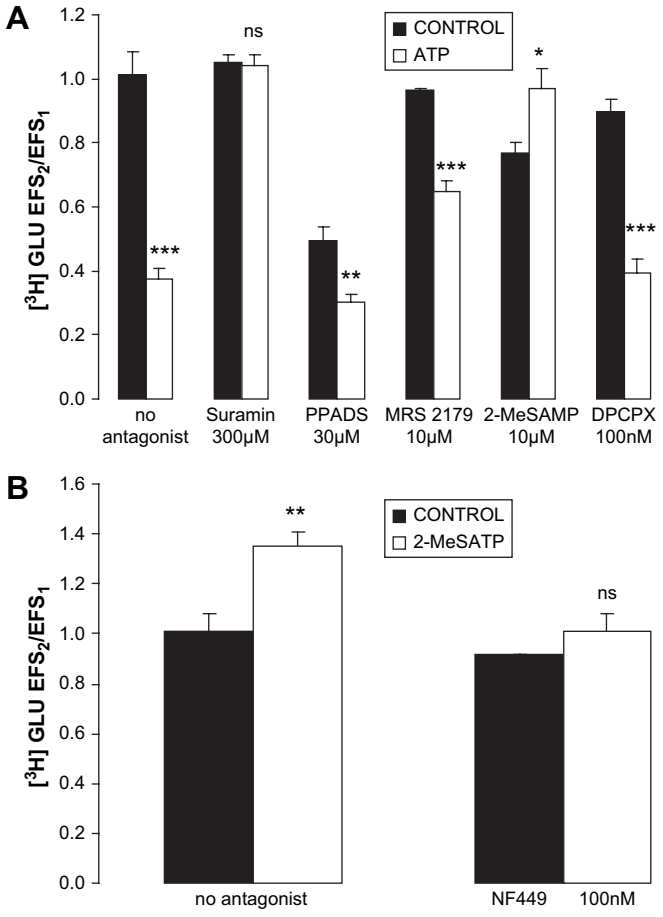

Fig. 3. The effect of different P1 and P2 receptor antagonists on the modulation of [^{3}H]GLU release by ATP (A) and 2-MeSATP (B). The bars show the EFS_2/EFS_1 ratios of tritium overflow in the absence (control) or presence of ATP (A) and 2-MeSATP (B), in the absence (no antagonist) or presence of the indicated antagonist. A. The inhibitory effect of ATP was reversed by suramin (300 μM) and by the $P2Y_{12,13}$ receptor antagonist 2-methylthioadenosine 5′-monophosphate (2-MeSAMP, 10 μM), and partly by the $P2Y_1$ receptor antagonist 2′-deoxy-N^6-methyladenosine 3′,5′-diphosphate diammonium salt (MRS 2179, 10 μM) and 4-[[4-Formyl-5-hydroxy-6-methyl-3-[(phosphonooxy) methyl]-2-pyridinyl]azo]-1,3-benzenedisulfonic acid tetrasodium salt (PPADS, 30 μM). The effect of ATP (300 μM) on evoked tritium overflow did not change when the P1(A_1) receptors were blocked by 100 nM 8-cyclopentyl-1,3-dipropylxanthine (DPCPX). B. 4,4′,4″,4‴-[carbonylbis[imino-5,1,3-benzenetriyl bis (carbonyl-imino)]] tetrakis (benzene-1,3-disulfonic acid) octasodium salt (NF449, 100 nM) prevented the facilitatory effect of 2-MeSATP (300 μM) on [^{3}H]GLU release. Agonists were perfused from 18 min before the second stimulation (S_2) and thereafter, whereas antagonists were preperfused from 18 min before the first stimulation period (S_1) until the end of the sample collection period. The values show the mean ± SEM of 3–8 identical experiments. Asterisks indicate significant differences from the respective controls, calculated by the Student's *t*-test (D). (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

$P < 0.01$, vs. ATP alone, Fig. 3A). When the slices were preperfused with the selective P1 (A_1)-adenosine receptor antagonist DPCPX (100 nM), the effect of ATP was not changed (Fig. 3A). The facilitatory effect of 2-MeSATP (300 μM) was reversed by the $P2X_1$ receptor selective antagonist NF449 (100 nM, Fig. 3B). When perfused alone between the first (EFS_1) and second (EFS_2) stimulation period, PPADS and 2-MeSAMP slightly decreased, whereas suramin, MRS 2179, and NF449 had no significant effect on the stimulation-evoked [^{3}H]GLU efflux (Table 2).

Since [^{3}H]GLU efflux, measured from the acute rat spinal cord slices could be derived either from primary excitatory nerve terminals, or from intrinsic interneurons, we envisaged the possibility that the effect of P2Y receptor activation on [^{3}H]GLU efflux is indirect; and is mediated by excitatory or inhibitory neurotransmission. Blockade of excitatory neurotransmission by the *N*-methyl-D-aspartate (NMDA) receptor antagonist AP-5 (10 μM) and the non-NMDA receptor antagonist CNQX (10 μM) significantly decreased EFS-evoked [^{3}H]glutamate release, showing that intrinsic excitatory neurotransmission contributes to the efflux of [^{3}H]GLU under our experimental conditions (Table 3). CNQX + AP-5 also decreased basal [^{3}H]glutamate release (Table 3). When the effect of ATP (1 mM) was examined in the presence of CNQX + AP-5, its inhibitory effect on evoked [^{3}H]glutamate release was abolished, and, instead, a net facilitation was detected (Table 3). By contrast, the γ-aminobutyric acid (GABA) A receptor antagonist bicuculline (100 μM) did not affect the inhibitory effect of ATP on the release of [^{3}H]GLU (data not shown).

3.2. [^{3}H]NA release experiments

Similarly to [^{3}H]GLU release experiments, basal tritium efflux was constant after preloading the spinal cord slices with [^{3}H]NA (0.437 ± 0.015%, $n = 8$), and electrical field stimulation (40 V, 15 Hz, 3.5 ms, 1 min) elicited a rapid and reproducible increase in tritium efflux, with an EFS_2/EFS_1 ratio of 0.93 ± 0.03 ($n = 8$) (Fig. 4A). Previous studies demonstrated that electrical field-stimulation evoked [^{3}H]NA efflux under identical conditions is $[Ca^{2+}]_o$-dependent (Uchihashi et al., 1998).

Among P2 purinoceptor agonists, ATP, ADP and 2-MeSADP decreased the stimulation-evoked [^{3}H]NA outflow in a narrow concentration range (Figs. 4B and 5), with the following rank order of agonist potency: ADP ≥ 2-MeSADP > ATP (Fig. 5). In case of ADP and 2-MeSADP the maximal effect was obtained at 30 μM concentration (43.7 ± 8.1% and 32.2 ± 1.8% inhibition, $n = 4$), and when higher concentrations were examined, less inhibition was detected (Fig. 5). These agonists did not significantly affect basal release (data not shown).

Just as in the case of [^{3}H]GLU release experiments, 2-MeSATP did not inhibit, but in fact significantly enhanced the stimulation-evoked efflux of [^{3}H]NA in the concentration range of 100–300 μM (Figs. 4C and 5). In addition, 2-MeSATP also increased the basal efflux of [^{3}H]NA (Fig. 4C, at 300 μM: 0.987 ± 0.036%, $n = 4$, $P < 0.01$ vs. control).

The effect of ATP was prevented by the $P2Y_{12,13}$ receptor antagonist 2-MeSAMP (10 μM) and, similarly to [^{3}H]GLU experiments, a net potentiation was observed (Fig. 6A). When the slices were preperfused with MRS 2179 (10 μM), and DPCPX (100 nM), the effect of ATP was partly reversed (Fig. 6A). On the other hand, suramin (300 μM) and PPADS (30 μM), non-selective antagonists of P2 receptors, did not affect the inhibitory

Table 2
Effect of P2 receptor antagonists on electrically evoked tritium overflow from rat spinal cord

Drugs (μM)	[^{3}H]glutamate			[^{3}H]noradrenaline		
	EFS_2/EFS_1	n	Significance	EFS_2/EFS_1	n	Significance
CTRL	1.01 ± 0.07	8		0.92 ± 0.03	8	
Suramin (300)	1.19 ± 0.04	4	$P > 0.05$	1.17 ± 0.02	8	$P < 0.01$
PPADS (30)	0.78 ± 0.03	4	$P < 0.05$	0.74 ± 0.01	4	$P < 0.05$
MRS 2179 (10)	0.95 ± 0.04	4	$P > 0.05$	0.95 ± 0.04	8	$P > 0.05$
2-MeSAMP (10)	0.60 ± 0.01	4	$P < 0.01$	0.93 ± 0.02	4	$P > 0.05$
NF449 (0.1)	0.89 ± 0.02	4	$P > 0.05$	0.81 ± 0.03	4	$P > 0.05$

Field stimulation was used twice, 30 min apart, with 40 V, 15 Hz, 3.5 ms, and 1 min ([^{3}H]glutamate) and 40 V, 3 Hz, 1 ms, and 2 min ([^{3}H]noradrenaline). P2 receptor antagonists were added between the first (S_1) and second (S_2) stimulations, 18 min prior to the second stimulation, at concentrations given in parentheses. Changes in the EFS_2/EFS_1 ratio represent the effect of drugs on the stimulation-evoked release of [^{3}H]glutamate and [^{3}H]noradrenaline. Data show the mean ± SEM of n observations. P values indicate significant differences from respective controls (CTRL), calculated by one-way analysis of variance followed by Dunnett's test.

effect of ATP (Fig. 6A). The facilitatory action of 2-MeSATP (300 μM) on [^{3}H]NA efflux was sensitive to inhibition by the $P2X_1$ receptor selective antagonist NF449 (100 nM) and by PPADS (30 μM) (Fig. 6B). By contrast, the facilitatory effect of 2-MeSATP (100 μM) persisted in the presence of $P2Y_1$ receptor selective antagonist MRS 2179 (10 μM) (Fig. 6B); moreover, it was potentiated (21.38 ± 0.33% and 106.63 ± 2.7% facilitation in the absence and presence of MRS 2179, respectively, $n = 4$, $P < 0.0001$). The antagonists used in these experiments did not significantly change the [^{3}H]NA overflow evoked by stimulation, with the exception of suramin and PPADS (Table 2), the former enhancing and the latter slightly decreasing the evoked tritium efflux.

3.3. RT-PCR study in the rat brainstem, DRG, and spinal cord

Since the pharmacological analysis suggested that the receptor subtypes responsible for inhibitory modulation of [^{3}H]GLU and [^{3}H]NA release might include the $P2Y_{12}$ or $P2Y_{13}$ receptor, RT-PCR analysis was performed in order to confirm whether mRNAs encoding $P2Y_{12}$ and $P2Y_{13}$ receptors are expressed in those areas where cell bodies of the neurons releasing noradrenaline and glutamate in the spinal cord are localized. Thus, we examined mRNA expression of $P2Y_{12}$ and $P2Y_{13}$ receptor subunits in the in the rat DRG and spinal cord, the areas containing the cell bodies of glutamatergic primary afferent neurons and spinal interneurons, which are the potential source of [^{3}H]GLU release in the spinal cord and in the rat brainstem, where cell bodies of the descending noradrenergic pathway projecting to the spinal cord are situated. Total RNA samples were reverse transcribed and amplified by PCR using primers specific to the two different P2Y receptor subtypes. RT-PCR analysis showed positive signals for both $P2Y_{12}$- and $P2Y_{13}$-receptors in the rat brainstem (Fig. 7A). These bands were absent when the reverse transcriptase was omitted, and were thus not due to the presence of contaminating genomic DNA. We then explored the mRNA expression of P2Y receptor subunits in the spinal cord. RT-PCR analysis revealed that the size of the $P2Y_{13}$-specific amplification product

Table 3
Effect of the *N*-methyl-D-aspartate (NMDA) receptor antagonist D(−)-2-amino-5-phosphonopentanoic acid (AP-5) and the non-NMDA receptor antagonist 6-cyano-7-nitroquinoxaline-2,3-dione-disodium (CNQX) on basal and electrically evoked tritium overflow from rat spinal cord and on the adenosine 5′-triphosphate (ATP)-mediated inhibition of evoked [^{3}H]glutamate ([^{3}H]GLU) release

	Control		+CNQX + AP-5		
	[^{3}H]GLU	n	[^{3}H]GLU	n	Significance
Basal outflow (%)	3.097 ± 0.160	8	2.013 ± 0.062[a]	4	$P < 0.0001$
EFS_2 (%)	6.016 ± 0.040	8	0.613 ± 0.050[a]	4	$P < 0.0001$
EFS_2/EFS_1					
−	1.013 ± 0.070	8	0.269 ± 0.020[a]	4	$P < 0.0001$
			0.613 ± 0.050[b]	4	
+ATP (1 mM)	0.373 ± 0.032**	8	1.377 ± 0.038[b]**	4	$P < 0.0001$

Tissue preparations were incubated with [^{3}H]GLU for 30 min and electrically stimulated twice (S_1, S_2; 40 V, 15 Hz, 3.5 ms, 1 min). Basal efflux of tritiated glutamate was measured in a 3-min sample, collected immediately before the second stimulation period. Electrical stimulation-induced [^{3}H]GLU efflux (EFS_2) was expressed by calculating the net release in response to electrical stimulation by the area-under-the-curve method − i.e. by subtracting the release before the electrical stimulation from the values measured after stimulation. Changes in the EFS_2/EFS_1 ratio represent the effect of drugs on stimulation-evoked release of [^{3}H]GLU. CNQX + AP-5 was added between the first (S_1) and second (S_2) stimulations, 21 min prior to the second stimulation in the experiments indicated by [a], whereas CNQX + AP-5 was present during both S_1 and S_2 in the experiments indicated by [b]. ATP was added between the first (S_1) and second (S_2) stimulations, 18 min prior to the second stimulation. The values show means ± SEM from n identical experiments. P values indicate significant differences between CNQX + AP-5-treated (+CNQX + AP-5) and control groups, assessed by Student's t-test. Asterisks indicate significant difference between ATP-treated slices and the respective ATP-free controls (−), $^{**}P < 0.0001$.

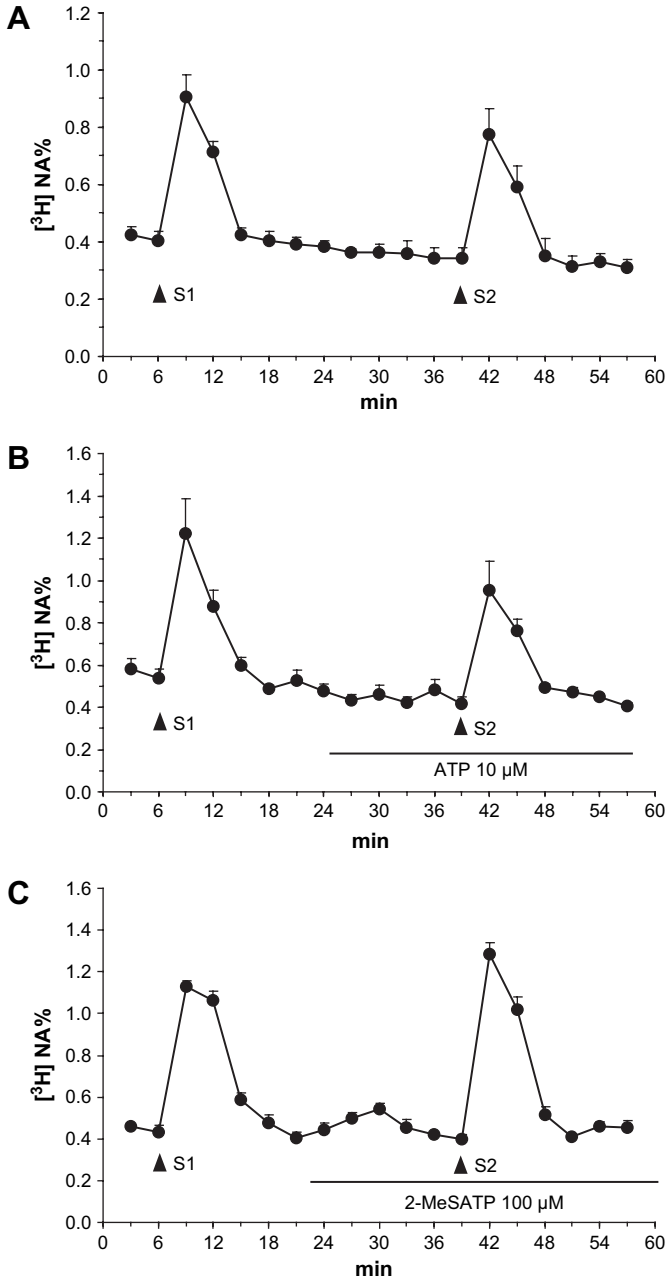

Fig. 4. Modulation of electrical field stimulation-induced [^{3}H]noradrenaline ([^{3}H]NA) release by nucleotides in superfused rat spinal cord slices. A. Basal and electrical field stimulation-induced [^{3}H]NA efflux in control experiments. After preperfusion, the slices were stimulated electrically (S_1, S_2). [^{3}H]NA release is expressed as a percentage of the amount of radioactivity in the tissue at the sample collection time (fractional release, %). B–C. The effect of B. ATP (10 µM) and C. 2-MeSATP (100 µM) on the electrical stimulation-induced [^{3}H]NA release. A horizontal line indicates the presence of ATP or 2-MeSATP. Data show the mean ± SEM of 4–8 identical experiments.

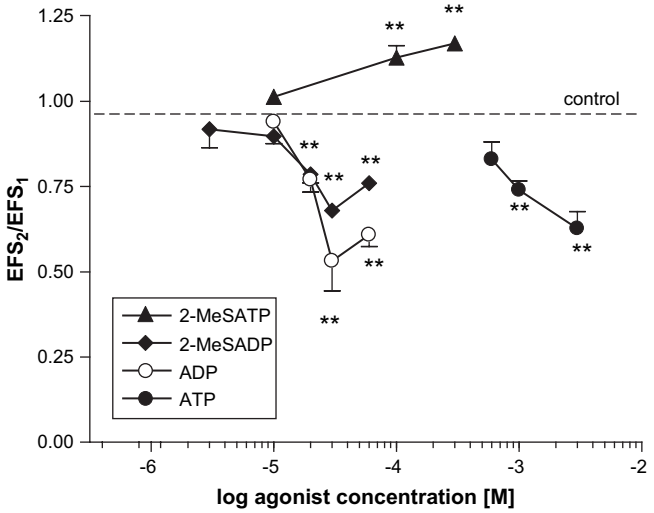

Fig. 5. Concentration-response relationship of the effect of ATP, 2-MeSADP, ATP and 2-MeSATP on electrical stimulation evoked [^{3}H]NA efflux. ATP (1 µM to 3 mM), 2-methylthioadenosine 5′-diphosphate (2-MeSADP, 3 µM to 60 µM), and adenosine 5′-diphosphate (ADP, 10 µM to 60 µM) concentration-dependently decreased the electrical stimulation-evoked [^{3}H]NA efflux from superfused rat spinal cord slices with the following rank order of agonist potency: ADP ≥ 2-MeSADP > ATP. 2-MeSATP (100 µM to 300 µM) exerted a facilitatory effect on evoked [^{3}H]NA efflux. The results are expressed as EFS_2/EFS_1 ratios in the presence of various concentrations of agonists indicated on the abscissa. A dotted line represents the control value. Data show the mean ± SEM of 4–8 identical experiments. Asterisks indicate significant differences from the respective control, calculated by one-way analysis of variance, followed by the Dunnett test (**$P < 0.01$).

was consistent with the expected sequence-based product size, indicating the expression of this P2 receptor subtype in the rat spinal cord (Fig. 7B). Similarly, we were able to detect mRNA expression of the $P2Y_{13}$ receptor in the rat DRG (Fig. 7C). Conversely, we were unable to demonstrate mRNA expression of the $P2Y_{12}$ receptor, either in the rat spinal cord or in the DRG (Figs. 7B,C).

3.4. $P2Y_1$ receptor immunohistochemistry

The pharmacological analysis also indicated that in addition to $P2Y_{12}$ and $P2Y_{13}$ receptors, the inhibitory effect of P2 receptor agonists on [^{3}H]GLU and [^{3}H]NA release might be also mediated by $P2Y_1$ receptors. However, because previous studies have already clarified that mRNA encoding $P2Y_1$ receptors is expressed in the rat brainstem, DRG and spinal cord respectively (Xiao et al., 2002; Papp et al., 2004; Kobayashi et al., 2006), we did not examine the expression of this receptor at the mRNA level. Instead, to explore the cell-type specific distribution of the $P2Y_1$ receptor protein and to reveal whether $P2Y_1$ receptors involved in the modulation of [^{3}H]GLU release are expressed on primary afferent nerve terminals or interneurons, immunohistochemical experiments were performed using a specific antibody raised against $P2Y_1$ receptors. In order to visualize glutamatergic nerve terminals in the spinal cord, immunostaining selective for VGLUT1 was also performed. Similarly to the recently published results of Persson and coworkers (Persson et al., 2006), immunoreactivity of VGLUT1 was present in different densities, but basically in the whole dorsal horn from lamina I–VI at the cervical level (Fig. 8A). As depicted in Fig. 8B at the electron microscopic level, DAB staining showed VGLUT1 immunoreactivity in the membrane of clear vesicles of glutamatergic terminals.

Although we cannot exclude a potential co-localization, the distribution pattern of $P2Y_1$ receptor immunolabeling in the

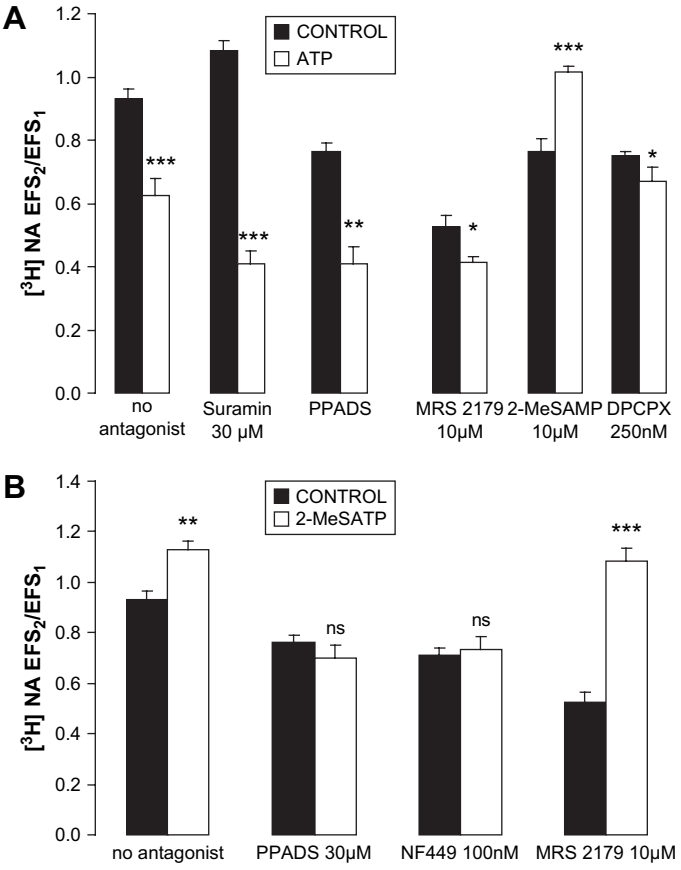

Fig. 6. The effect of different P1 and P2 receptor antagonists on the modulation of [^{3}H]NA release by ATP (A) and 2-MeSATP (B). Data show the EFS_2/EFS_1 ratios of tritium overflow in the absence (control) or presence of ATP, in the absence (no antagonist) or presence of the indicated antagonist. A. The P2 receptor agonist ATP (3 mM) significantly decreased the evoked [^{3}H]NA efflux. This inhibitory effect was reversed by the $P2Y_{12,13}$ receptor antagonist, 2-MeSAMP (10 µM), and partly by the $P2Y_1$ receptor antagonist MRS 2179 (10 µM), and the P1 receptor antagonist DPCPX (100 nM) but not by suramin (300 µM) and PPADS (30 µM). B. The facilitatory effect of 2-MeSATP (100 µM) on [^{3}H]NA release was antagonized by PPADS (30 µM) and by the $P2X_1$ receptor selective antagonist NF449 (100 nM). By contrast, the $P2Y_1$ receptor antagonist MRS 2179 (10 µM) had no effect on 2-MeSATP-evoked facilitation of [^{3}H]NA release. P2 and P1 receptor agonists were perfused from 18 min before the second stimulation (S_2) and thereafter, whereas antagonists were preperfused from 18 min before the first stimulation period (S_1) until the end of the sample collection period. Data show the mean ± SEM of 3–8 identical experiments. Asterisks indicate significant differences from the respective control, calculated by the Student's *t*-test. (*$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$).

transverse cervical section was different from that of VGLUT1 staining. The most intense $P2Y_1$ staining was found in lamina I–II, and the density of immunostaining weakened in the medial part of the dorsal horn (Fig. 8C). Electron histochemical staining for $P2Y_1$ receptor protein revealed immunoreactive dendrites (Fig. 8D) but not synapses. According to our previous study, DAB precipitates indicating the presence of the $P2Y_1$ receptor were also observable on the luminal membrane of endothelial cells, presumably due to the caveolae docking here (Fig. 8E) (Kittel et al., 2004).

4. Discussion

4.1. Inhibitory P2Y receptors

Previous results suggested that the release of glutamate from the hippocampus is subject to inhibitory modulation by $P2Y_1$ receptors (Rodrigues et al., 2005) and that of noradrenaline from sympathetic nerves by $P2Y_{12}$ and/or $P2Y_{13}$ receptors (Queiroz et al., 2003; Lechner et al., 2004). The mechanism of P2Y receptor mediated inhibition of noradrenaline release has also been explored: the activation of P2Y receptors inhibits voltage dependent Ca^{2+} influx and thereby limits the Ca^{2+} dependent vesicular exocytosis and subsequent efflux of noradrenaline to the extracellular space (Powell et al., 2000; Kulick and von Kugelgen, 2002; Lechner et al., 2004). A similar P2Y receptor-mediated inhibitory control of noradrenaline release has also been reported in central catecholaminergic pathways innervating the cerebral cortex and the hippocampus (von Kügelgen et al., 1994; Koch et al., 1997), although the underlying receptor subtype has not been identified in these studies.

P2Y receptors are classified according to their sensitivity to purines and/or pyrimidines: $P2Y_{1,12}$ and $P2Y_{13}$ are adenine nucleotide-preferring receptors; $P2Y_6$ is preferred by uridine nucleotides; $P2Y_{2,4}$ and $P2Y_{11}$ are receptors with mixed selectivity; whereas $P2Y_{14}$ is activated only by UDP-glucose, UDP-galactose, UDP-N-acetylglucosamine and UDP-glucuronic acid (Abbracchio et al., 2006). In our experiments, ATP, ADP and its analog 2-MeSADP were all effective at inhibiting [^{3}H]GLU efflux from the spinal cord, with the following rank order of agonist potency: ADP > 2-MeSADP > ATP, whereas 2-MeSATP exhibited a biphasic effect on stimulation evoked [^{3}H]GLU efflux with a predominant facilitation. These compounds are agonists at $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ receptor subtypes, and the following order of agonist potency has been established among them: $P2Y_1$: 2-MeSADP = 2-MeSATP > ADP > ATP; $P2Y_{12}$: 2-MeSADP > ADP > ATP; and $P2Y_{13}$: ADP > 2-MeSADP ≫ ATP (cf. von Kügelgen et al., 2006).

Therefore, the inhibitory effect of ATP on glutamate release is most likely mediated by $P2Y_{13}$ receptors. This assumption is supported by agonist potency order and by the antagonism of the inhibitory effect of ATP by 2-MeSAMP, an antagonist predominantly acting at $P2Y_{12}$ and $P2Y_{13}$ receptors (von Kügelgen, 2006). However, the selective $P2Y_1$ receptor antagonist MRS 2179 also partially reversed the action of ATP. Since MRS 2179 does not inhibit P2Y subtypes other than the $P2Y_1$ receptor at the concentration used (von Kügelgen, 2006), it is likely that $P2Y_1$ receptors are also involved in the inhibitory modulation of glutamate release. The effect of ATP was fully antagonized by suramin (300 µM), which is compatible with the involvement of both $P2Y_1$ and $P2Y_{13}$ receptors. The inhibitory effect of ATP on [^{3}H]GLU release was completely sustained in the presence of DPCPX, indicating that the degradation of ATP to adenosine and its subsequent action on A_1 adenosine receptors does not participate in this action. For the same reason, the participation of $P2Y_1/A_1$ heteromeric receptors is also unlikely (Yoshioka et al., 2001).

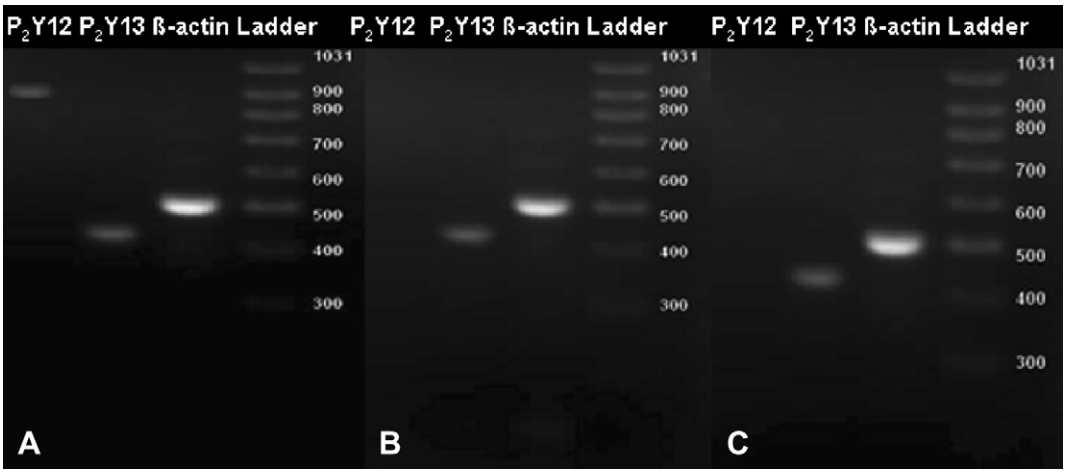

Fig. 7. mRNA expression of $P2Y_{12}$ and $P2Y_{13}$ receptors in the rat brainstem (A) dorsal root ganglion (DRG) (B) and spinal cord (C). To examine the involvement of various subtypes of purine receptors in the effect of purinoceptor agonists, mRNA expression of P2Y receptor subtypes was studied. Using primers specific for cDNAs of the given subtypes of P2Y receptors, reverse transcriptase-polymerase chain reaction (RT-PCR) analysis showed that mRNAs encoding $P2Y_{12}$ and $P2Y_{13}$ receptors were present in the rat brainstem (A), and that mRNA encoding $P2Y_{13}$, but not $P2Y_{12}$ receptors was expressed in the rat DRG (B) and spinal cord (C). Amplification of β-actin was used as an internal control and a 100-base pair DNA ladder (Fermentas, Vilnius, Lithuania) was used to identify PCR fragment sizes. The gels (1.5%, 1 × Tris/Borate/EDTA (TBE) buffer) shown are representative of at least two independent experiments.

When the release of [^{3}H]NA efflux was measured, although a similar pharmacological profile was obtained, subtle differences were also exhibited. Among P2 receptor agonists ATP, ADP and 2-MeSADP all decreased electrically evoked tritium overflow, but with a slightly different rank order of agonist potency: ADP $\geq$ 2-MeSADP $>$ ATP. This finding indicated that $P2Y_{13}$ receptors might predominantly mediate this inhibitory effect, but because ADP was relatively less potent than in case of [^{3}H]GLU release experiments, other subtypes of the P2Y receptor family such as the $P2Y_1$ and the $P2Y_{12}$ receptors might also contribute to this effect. This assumption is supported by the lack of antagonism by PPADS, which is an antagonist acting at $P2Y_1$ and $P2Y_{13}$ receptors, but not at $P2Y_{12}$ receptors and by the partial reversal of the action of ATP by MRS 2179. However, in our hands, suramin, which is an antagonist at both $P2Y_1$ and $P2Y_{12}$ receptors, failed to reverse the effect of ATP. The only known P2Y receptor subtype, which is insensitive to suramin is the $P2Y_4$ receptor. However, ADP, the most potent agonist in our experiments is inactive at the $P2Y_4$ receptor (Nicholas et al., 1996), which argues against the major role of $P2Y_4$ receptors in the inhibition of [^{3}H]NA release. For the same reason, the involvement of $P2Y_2$, $P2Y_6$ and $P2Y_{14}$ receptors are also unlikely, whereas the involvement of $P2Y_{11}$ receptors can be excluded, because there is no rodent orthologue of this receptor. Although the effect of ATP was partially sensitive to DPCPX, which is in line with the involvement of the A_1/$P2Y_1$ heteromeric receptor, or a pure A_1 adenosine receptor, these latter receptors are not sensitive to the antagonists, which fully or partially reversed the action of ATP (MRS 2179, 2-MeSAMP, Yoshioka et al., 2001). Finally, 2-MeSAMP, an antagonist, relatively selective to $P2Y_{12}$ and $P2Y_{13}$ receptors again fully reversed the effect of ATP indicating that at least one of these two subtypes are involved in the inhibitory modulation of [^{3}H]NA efflux. Nevertheless we cannot exclude, that the activation of an unknown subtype of P2Y receptor or a combination or a heteromeric association of a set of different P2Y receptors are responsible for the inhibition of [^{3}H]NA efflux under our experimental conditions, which results in a mixed pharmacological profile. One should also bear in mind that the ligand binding profile of P2Y and P2X receptors are overlapping, therefore co-activation of different subtypes of P2X and P2Y receptors in a native tissue may not mirror adequately the receptor profile obtained under the conditions of recombinant systems.

Supporting the conclusion drawn from the pharmacological data, RT-PCR analysis confirmed that mRNAs encoding $P2Y_{12}$ and $P2Y_{13}$ receptors are expressed in the rat brainstem, where cell bodies of the descending noradrenergic pathway are situated, whereas mRNA encoding the $P2Y_{13}$, but not $P2Y_{12}$ receptor was present in the rat DRG and in the spinal cord, where cell bodies of neurons releasing glutamate in the spinal cord are located. This arrangement is consistent with the presence of functional $P2Y_{12}$ receptors in brainstem catecholaminergic nuclei (Laitinen et al., 2001). As for $P2Y_1$ receptors, previous studies have already convincingly demonstrated their expression at the mRNA and protein levels in the brainstem (Moran-Jimenez and Matute, 2000; Moore et al., 2001; Papp et al., 2004) and at the mRNA level in the spinal cord (Kobayashi et al., 2006) and DRG (Xiao et al., 2002; Ruan and Burnstock, 2003; Kobayashi et al., 2006). In order to identify the cellular localization of $P2Y_1$ receptors responsible for the inhibition of glutamate release in the spinal cord, immunohistochemical experiments were also performed. Previous studies showed that the $P2Y_1$ receptor protein is expressed in acutely dissected (Ruan and Burnstock, 2003) and cultured (Gerevich et al., 2004) DRG neurons and suggested that the receptor protein is also localized to the central terminals of these cells. In spite of these data, we were unable to detect $P2Y_1$ receptor immunoreactivity in the axon terminals of the spinal cord, examined either by light or electron microscopy. Although the

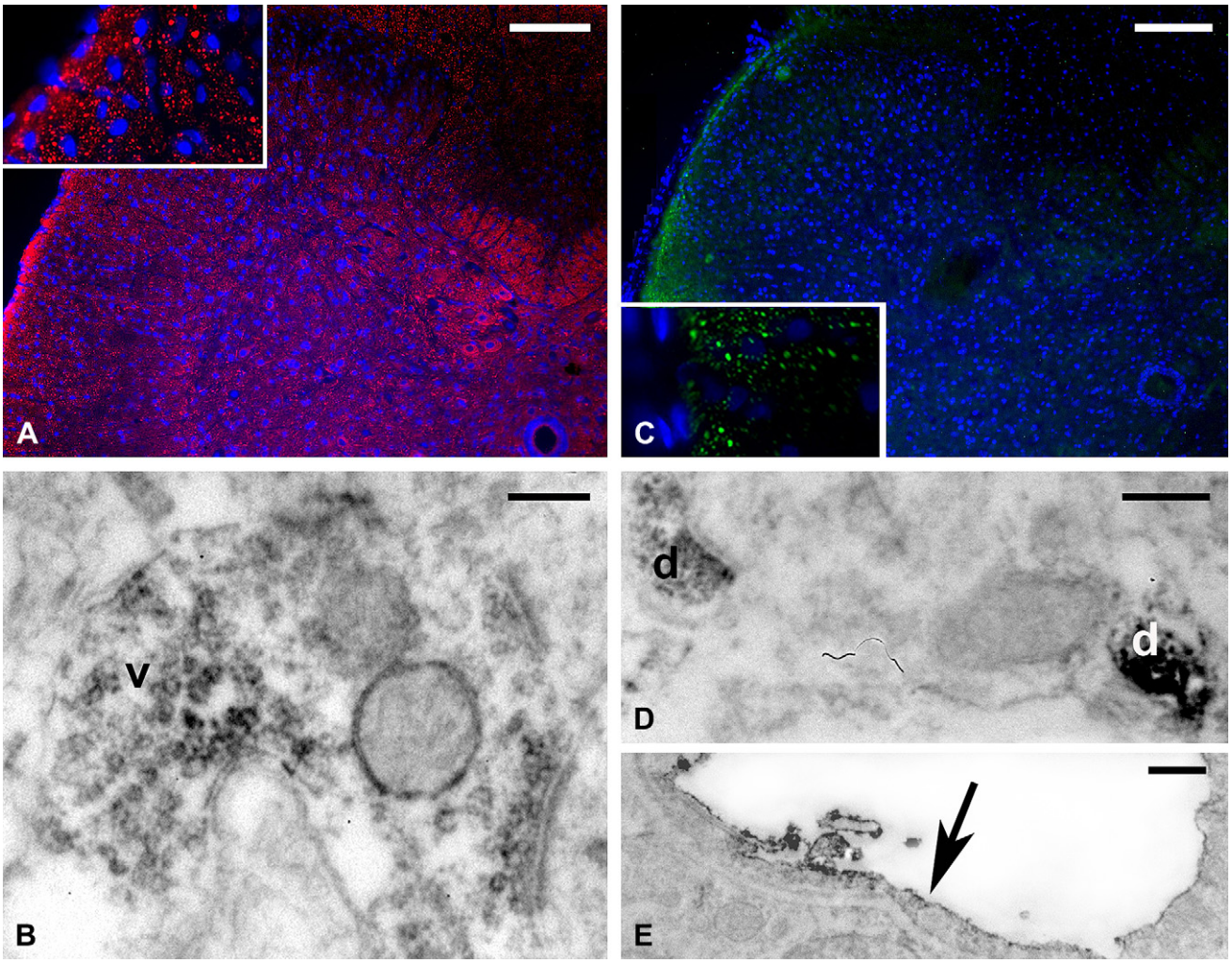

Fig. 8. Demonstration of the immunoreactivity specific for vesicular glutamate transporter 1 (VGLUT1), and $P2Y_1$, receptor in the rat spinal cord at the cervical level. A. Transverse section of rat spinal cord. Immunofluorescence staining for VGLUT1. Glutamatergic nerve terminals are labeled with Alexa 594 (red). Immunoreactivity is visible from lamina I–VI in the dorsal horn. DAPI staining (blue) indicates the nucleus. wm, white matter; bar, 5 μm. B. VGLUT1 staining at the electron microscopic level. Labeled vesicles (v) in a glutamatergic nerve terminal of the dorsal horn. ABC–DAB staining. Bar, 0.5 μm. C. Immunofluorescence staining for the $P2Y_1$ receptor in the dorsal horn of the rat spinal cord (transverse section). Labeling is mainly in lamina I–II. Immunofluorescence staining was carried out using the anti-rabbit Alexa 488 antibody (green) and DAPI staining (blue) for the nucleus. wm, white matter; bar, 5 μm. D. ABC–DAB staining demonstrates $P2Y_1$ immunoreactive dendrites (d) in the cervical section of the rat spinal cord. E. $P2Y_1$ immunoreactivity in the luminal membrane of the endothelial cell (arrow). ABC–DAB staining. Bar, 0.5 μm. F. Pictures were taken under a Nikon Eclipse E600 microscope equipped with a SPOT RT color digital camera, and captured images were printed using Adobe Photoshop 8.0 Software (A, C). Electron microscopic images (B, D, E) were taken using a Hitachi 2001 transmission electron microscope.

reason for this apparent divergence is not known, the expression pattern of receptors is not necessarily the same at cell bodies, situated in the DRG, and at their central terminals, which are in the spinal cord. By contrast, we identified $P2Y_1$ receptor immunoreactivity on the dendrites in the dorsal horn of the spinal cord, which showed different distribution pattern from VGLUT1 immunoreactivity, the marker of glutamatergic nerve terminals. Hence, $P2Y_1$ receptors, presumably responsible for the modulation of glutamate efflux, are localized to interneurons in the spinal cord. To support this notion, when excitatory neurotransmission were blocked by glutamate receptor antagonists, the inhibitory modulation of glutamate efflux was eliminated, indicating that the receptor is located downstream from the primary afferent synapse, probably on the dendrites of excitatory interneurons.

Since there is growing evidence that ATP, acting on P2Y receptors could modulate the action of glutamate on NMDA receptors (Luthardt et al., 2002; Resende et al., 2007), we cannot exclude the direct inhibitory interaction of P2Y receptors with pre- or postsynaptic NMDA receptors either.

Since the inhibitory modulation of noradrenaline and glutamate release displayed a similar pharmacological pattern and glutamate is able to release noradrenaline by the activation of NMDA receptors in the spinal cord, (Sundstrom et al., 1998; Nakai et al., 1999), it is a likely possibility that the release of both noradrenaline and glutamate is under the regulatory influence of the same P2Y receptors, which are present on dendrites of intrinsic excitatory neurons. This assumption, although supported by the findings of the immunohistochemical experiment of the present study, needs further investigation.

Our findings are compatible with a role for P2Y receptors in the inhibition of slow depolarization of substantia gelatinosa neurons induced by repetitive stimulation of the dorsal root (Yoshida et al., 2002). Moreover, they corroborate the observation that the $P2Y_{1,12,13}$ receptor agonist ADP-β-S inhibits polysynaptic but not monosynaptic, excitatory postsynaptic potentials in the hemisected spinal cord and exhibits antinociceptive potential in the tail flick test (Gerevich et al., 2004), although in this latter study the response to ADP-β-S was not antagonized by either PPADS or the $P2Y_{12,13}$ antagonist AR-C69931MX (cangrelor), and therefore was classified as being mediated by an unknown subtype of P2Y receptor.

4.2. Facilitatory P2X receptors

Interestingly, 2-MeSATP in the high micromolar concentration range enhanced glutamate and noradrenaline release in the rat spinal cord. This observation does not support the notion that the $P2Y_1$ receptor is the predominant inhibitory receptor regulating noradrenaline and glutamate release, because 2-MeSATP is a full, potent agonist at the $P2Y_1$ receptor (Waldo and Harden, 2004), whereas it is a weak, partial agonist at the $P2Y_{13}$ receptor (Marteau et al., 2003; von Kügelgen, 2006). The net facilitation of both basal and stimulation-evoked release, is rather indicative of the participation of a facilitatory ionotropic receptor in these effects. Moreover, the findings that the effect of 2-MeSATP was potentiated by MRS 2179, and that ATP also exhibited a facilitatory effect in the presence of 2-MeSAMP, indicate that that these agonists co-activate P2Y and a facilitatory receptor, and that the inhibitory action of ATP on P2Y receptors prevails over facilitatory receptor activation. The stimulatory effect of 2-MeSATP on glutamate and noradrenaline release was antagonized by the $P2X_1$ receptor selective antagonist NF449, and the facilitatory modulation of release noradrenaline was also inhibited by PPADS but not by MRS 2179. Although these effects have not been characterized in detail, they are compatible with the involvement of the $P2X_1$ receptor or a hetero-oligomeric receptor containing the $P2X_1$ subunit (such as $P2X_{1/5}$) and could reflect the presynaptic facilitation of glutamatergic excitatory postsynaptic currents in the rat spinal cord by the activation of $P2X_{1/5}$ receptors (Gu and MacDermott, 1997; Nakatsuka and Gu, 2001; Nakatsuka et al., 2003). In fact, 2-MeSATP is a potent agonist at recombinant rat $P2X_1$ receptors, and it is more potent than α,β-methylene ATP (Gever et al., 2006). By contrast, because NF449 has a micromolar affinity for $P2X_3$ receptors, and even less for the P2Y receptors (Braun et al., 2001), these receptor subtypes are less likely to be involved in the facilitation of glutamate and noradrenaline efflux by 2-MeSATP.

Interestingly, blockade of excitatory neurotransmission turned the inhibitory effect of ATP on glutamate efflux into facilitation, which indicates that that $P2X_1$ receptors responsible for this facilitation are located on the excitatory nerve terminals themselves, consistently with electrophysiological observations (Gu and MacDermott, 1997; Nakatsuka and Gu, 2001; Nakatsuka et al., 2003).

4.3. Conclusion

We have demonstrated that P2 receptor agonists exert dual and opposite modulation on electrically evoked noradrenaline and glutamate release in the spinal cord of the rat. The inhibitory effect of ATP on glutamate release is mediated by the $P2Y_{13}$ receptor, and/or by $P2Y_1$ receptors, whereas the inhibition of noradrenaline release is most likely mediated by the $P2Y_{13}$ receptor and/or by $P2Y_1$ and $P2Y_{12}$ receptors. In addition, a stimulatory effect on both noradrenaline and glutamate release was also detected through activation of a $P2X_1$-like receptor. Our results therefore indicate that, in addition to P2X receptors (Burnstock, 2006b), sensory information processing and its modulation by the descending noradrenergic pathway in the spinal cord could be also targeted by distinct subtypes of P2Y receptors, providing novel sites of action for potential analgesic compounds.

Acknowledgments

The authors are grateful to Maria Baranyi for HPLC analyses. This study was supported by a grant from the Hungarian Research and Development Fund (NKFP1A/002/2004 to B.S.).

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