

RESEARCH PAPER

Facilitation of transmitter release from rat sympathetic neurons via presynaptic P2Y₁ receptors

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BACKGROUND AND PURPOSE

P2Y₁, P2Y₂, P2Y₄, P2Y₁₂ and P2Y₁₃ receptors for nucleotides have been reported to mediate presynaptic inhibition, but unequivocal evidence for facilitatory presynaptic P2Y receptors is not available. The search for such receptors was the purpose of this study.

EXPERIMENTAL APPROACH

In primary cultures of rat superior cervical ganglion neurons and in PC12 cell cultures, currents were recorded via the perforated patch clamp technique, and the release of [³H]-noradrenaline was determined.

KEY RESULTS

ADP, 2-methylthio-ATP and ATP enhanced stimulation-evoked ³H overflow from superior cervical ganglion neurons, treated with pertussis toxin to prevent the signalling of inhibitory G proteins. This effect was abolished by P2Y₁ antagonists and by inhibition of phospholipase C, but not by inhibition of protein kinase C or depletion of intracellular Ca²⁺ stores. ADP and a specific P2Y₁ agonist caused inhibition of Kv7 channels, and this was prevented by a respective antagonist. In neurons not treated with pertussis toxin, ³H overflow was also enhanced by a specific P2Y₁ agonist and by ADP, but only when the P2Y₁₂ receptors were blocked. ADP also enhanced K⁺-evoked ³H overflow from PC12 cells treated with pertussis toxin, but only in a clone expressing recombinant P2Y₁ receptors.

CONCLUSIONS AND IMPLICATIONS

These results demonstrate that presynaptic P2Y₁ receptors mediate facilitation of transmitter release from sympathetic neurons most likely through inhibition of Kv7 channels.

Abbreviations

2-MeSATP, 2-methylthio-ATP; DMSO, dimethyl sulphoxide; H-7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine; I_M, M-type K⁺ current; MRS 2179, 2'-deoxy-N6-methyladenosine 3',5'-bisphosphate tetrasodium salt; MRS 2365, [(1R,2R,3S,4R,5S)-4-[6-amino-2-(methylthio)-9H-purin-9-yl]-2,3-dihydroxybicyclo[3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt; PIP₂, phosphatidylinositol 4,5-bisphosphate; PTX, pertussis toxin; SCG, superior cervical ganglion; TTX, tetrodotoxin; U73122, 1-[6-(((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino)hexyl]-1H-pyrrole-2,5-dione

Introduction

ATP is released together with noradrenaline from sympathetic nerve terminals and contributes to the sympatho-effector transmission (von Kugelgen and Starke, 1991). This neurotransmission is tightly controlled by a large number of different presynaptic receptors, which also include autoreceptors for noradrenaline as well as nucleotides (Boehm and Kubista, 2002). Amongst the members of the adrenoceptor family, presynaptic α_{2A} and α_{2C} receptors mediate autoinhibition of noradrenaline release, whereas β_2 receptors mediate facilitation (Boehm and Kubista, 2002). Within the family of P2 receptors, ionotropic P2X receptors mediate facilitation of transmitter release, whereas metabotropic P2Y receptors were found to mediate inhibition only (Sperlagh *et al.*, 2007; Dorostkar and Boehm, 2008; Goncalves and Queiroz, 2008). In this respect, the family of P2Y receptors appear to differ from other GPCRs: most neurotransmitters and/or mediators, such as acetylcholine, adenosine, histamine, noradrenaline and prostaglandins are known to cause presynaptic inhibition as well as facilitation of sympathetic transmitter release, the two opposing actions being mediated by two different GPCRs (Boehm and Kubista, 2002). In general, presynaptic GPCRs linked to Gs or Gq type G-proteins mediate facilitation of noradrenaline release, whereas receptors linked to Gi/o-proteins mediate inhibition, although there are exceptions to this rule (Kubista and Boehm, 2006).

Within the family of G-protein-coupled P2Y receptors, at least eight different subtypes have been identified (P2Y₁, P2Y₂, P2Y₄, P2Y₆, P2Y₁₁, P2Y₁₂, P2Y₁₃, P2Y₁₄). In heterologous expression systems, all P2Y receptor subtypes, with the exception of P2Y₁₂, couple to PLC via Gq and mediate increases in inositol phosphates; via Gi/o, P2Y₁₂, P2Y₁₃ and P2Y₁₄ mediate inhibition, while P2Y₁₁ mediates activation of adenylyl cyclases (Abbracchio *et al.*, 2006; Burnstock, 2006). Accordingly, one might expect all P2Y but P2Y₁₂ receptors to act as facilitatory presynaptic P2Y receptors. However, with respect to the sub-classification of presynaptic P2Y receptors, the information available is limited, as most subtype selective ligands have been developed quite recently (Jacobson and Boeynaems, 2010). In fact, P2Y₁, P2Y₂, P2Y₄, P2Y₁₂ and P2Y₁₃ receptors have all been implicated in the presynaptic inhibition of transmitter release, but unequivocal evidence for facilitatory presynaptic P2Y receptors is lacking (Goncalves and Queiroz, 2008).

In sympathetically innervated tissues, such as the rat vas deferens, ADP and 2-methylthio-ADP inhibit [³H]-noradrenaline release, and this is prevented by 2-methylthio-AMP, an antagonist at P2Y₁₂ and P2Y₁₃ receptors (Queiroz *et al.*, 2003). Likewise, in PC12 cells and rat superior cervical ganglion (SCG) neurons, inhibitory presynaptic P2Y receptors were blocked by 2-methylthio-AMP and by the P2Y₁₂ antagonist cangrelor (Kulick & von Kugelgen, 2002; Lechner *et al.*, 2004). More recently, P2Y₁₂ together with P2Y₁ receptors were shown to mediate autoinhibition in sympathetically innervated tissues (Quintas *et al.*, 2009). Along the same line, evidence has been presented that P2Y₁, P2Y₁₂ and P2Y₁₃ receptors mediate inhibition of noradrenaline release in the central nervous system (Csolle *et al.*, 2008; Heinrich *et al.*, 2008). For this study, rat SCG neurons in primary cell culture as well as PC12 cells were used to

search for facilitatory presynaptic P2Y receptors. The results reveal that activation of presynaptic P2Y₁ receptors leads to an increase in sympathetic transmitter release through activation of PLC.

Methods

Cell cultures

Primary cultures of dissociated SCG neurons from neonatal rats were prepared as described previously (Boehm, 1999). Newborn Sprague-Dawley rats were kept and killed 3 to 10 days after birth by decapitation in full accordance with all rules of the Austrian animal protection law and the Austrian animal experiment bylaws. Ganglia were removed immediately after decapitation of the animals, cut into three to four pieces and incubated in collagenase (1.5 mg·mL⁻¹; Sigma, Vienna, Austria) and dispase (3.0 mg·mL⁻¹; Boehringer Mannheim, Vienna, Austria) for 20 min at 36°C. Subsequently, they were further incubated in trypsin (0.25% trypsin; Worthington, Lakewood, NJ) for 15 min at 36°C, dissociated by trituration and resuspended in Dulbeccos modified Eagle's Medium (Invitrogen, Lofer, Austria) containing 2.2 g·L⁻¹ glucose, 10 mg·L⁻¹ insulin, 25 000 IU·L⁻¹ penicillin and 25 mg·L⁻¹ streptomycin (Invitrogen), 50 µg·L⁻¹ nerve growth factor (R&D Systems Inc., Minneapolis, MN) and 5% fetal calf serum (Invitrogen). Finally, all cells were seeded onto 5 mm plastic discs for radiotracer release experiments and onto 35 mm culture dishes for electrophysiological experiments. The cultures were stored for 4 to 8 days in a humidified 5% CO₂ atmosphere at 36°C. On days 1 and 4 after dissociation, the medium was exchanged entirely.

PC12 cells were obtained from the European Collection of Cell Cultures (ECACC; Salisbury, UK) and kept in OptiMEM (Life Technologies, Vienna, Austria) supplemented with 0.2 mM L-glutamine (HyClone, Aalst, Belgium), 25 000 IU·L⁻¹ penicillin and 25 mg·L⁻¹ streptomycin (Sigma), 5% fetal calf serum and 10% horse serum (both Life Technologies). Once per week, cell cultures were split, and the medium was exchanged twice weekly. To investigate the release of previously incorporated [³H]-noradrenaline under continuous superfusion, PC12 cells were plated onto 5 mm discs, as described for the SCG neurons above. All tissue culture plastic was coated with rat tail collagen (Biomedical Technologies Inc., Stoughton, MA, USA).

PC12 cell clones stably expressing the rat P2Y₁ receptor linked to the green fluorescent protein (P2Y₁-GFP) were generated as described previously; here, cells of clone 8 were used (Moskvina *et al.*, 2003).

Determination of [³H]-noradrenaline release

[³H]-noradrenaline uptake and superfusion of SCG neurons and PC12 cells was performed as described previously (Lechner *et al.*, 2004). The plastic discs with dissociated neurons or PC12 cells were incubated in 0.05 µM [³H]-noradrenaline ((-)-[ring-2,5,6-³H]-noradrenaline, specific activity 1.369 TBq·mmol⁻¹; Perkin Elmer, Vienna, Austria) in culture medium supplemented with 1 mM ascorbic acid at 37°C for 1 h. Thereafter, the culture discs were transferred to small chambers and superfused with a buffer containing

(mM) NaCl (120), KCl (3.0), CaCl₂ (2.0), MgCl₂ (2.0), glucose (20), HEPES (10), fumaric acid (0.5), Na-pyruvate (5.0), ascorbic acid (0.57) and desipramine (0.001), adjusted to pH 7.4 with NaOH. Superfusion was performed at 25°C at a rate of about 1.0 mL·min⁻¹. Collection of 4 min fractions of superfusate was started after a 60 min washout period during which excess radioactivity had been removed.

Depolarization-dependent tritium overflow was triggered either by 36 monophasic rectangular electrical pulses (0.5 ms, 60 mA, 66 V·cm⁻¹) delivered at 0.3 Hz or by the inclusion of 25 mM KCl (NaCl was reduced accordingly to maintain isotonicity) in the buffer for periods of 120 s. These stimulations were started after 72 (S1) and 92 min (S2) of superfusion. Nucleotides and nucleotide receptor agonists or antagonists were included in the buffer from minute 88 onwards (see Figure 1). Tetrodotoxin (TTX), whenever appropriate, was included in the buffer after 50 min of superfusion (i.e. 10 min prior to the start of sample collection). The radioactivity remaining in the cells after the completion of experiments was extracted by immersion of the discs in 2% (v/v) perchloric acid followed by sonication. Radioactivity in extracts and collected fractions was determined by liquid scintillation counting (Packard Tri-Carb 2800 TR) with a counting efficiency of 63%. Radioactivity released in response to electrical

field stimulation from rat sympathetic neurons after labelling with tritiated noradrenaline under conditions similar to those of the present study had previously been shown to consist predominantly of the authentic transmitter and to contain only small amounts ($\leq 15\%$) of metabolites (Schwartz and Malik, 1993). Hence, the outflow of tritium measured in this study was assumed to reflect the release of noradrenaline and not that of metabolites.

The spontaneous (unstimulated) rate of ³H efflux was obtained by expressing the radioactivity retrieved during a collection period as percentage of the total radioactivity in the cultures at the beginning of this period. Stimulation-evoked tritium overflow was calculated as the difference between the total tritium outflow during and after stimulation and the estimated basal outflow that was assumed to follow a linear time course throughout experiments. Therefore, basal outflow during periods of stimulation was assumed to equate to the arithmetic mean of the samples preceding and those following stimulation, respectively. Differences between total and estimated basal outflow during periods of stimulation were expressed as percentages of total radioactivity in the cultures at the onset of stimulation (% of total radioactivity; %). The amount of radioactivity in the cultures at the beginning of each collection period is calculated

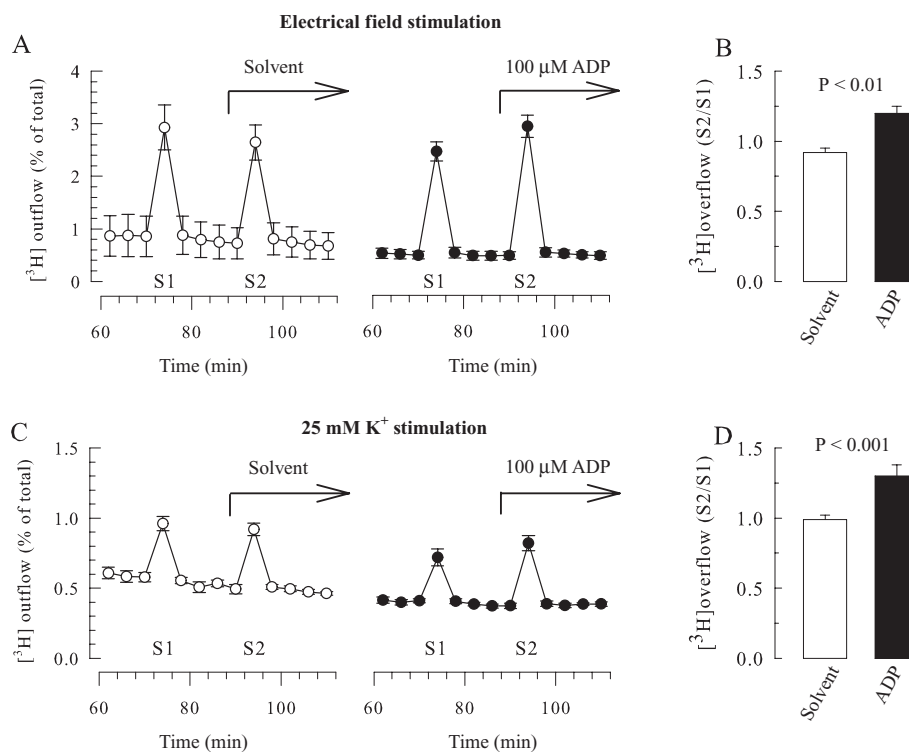


Figure 1

Enhancement of [³H]-noradrenaline release from rat SCG neurons treated with pertussis toxin by ADP. SCG cell cultures were treated with pertussis toxin (PTX; 100 ng·mL⁻¹ for 24 h) and were labelled with [³H]-noradrenaline and superfused. When appropriate (C and D), 0.1 μM TTX was present from minute 50 of superfusion onwards. Subsequent to a 60 min washout period, 4 min fractions of superfusate were collected. Tritium overflow was stimulated twice (S1 after 72 min and S2 after 92 min of superfusion) by 2 min exposures to either electrical pulses (A and B) or 25 mM KCl (C and D). Either 100 μM ADP or the appropriate solvent were present from minute 88 onwards, as indicated by the arrows in (A and C). (A and C) Exemplary time courses of fractional ³H outflow as a percentage of the total radioactivity in the cells (*n* = 3). (B and D) The S2/S1 ratios of tritium overflow evoked by electrical stimulation (B) or 25 mM K⁺ (D) in the presence of either solvent or 100 μM ADP (*n* = 11–12); the *P*-values for the statistical significance of differences (Student's unpaired *t*-test) are indicated above the bars.

by summing up the radioactivity remaining in the cells at the end of experiments and that retrieved during the respective and all subsequent collection periods.

As the amount of depolarization- or drug-induced tritium overflow may vary considerably between different cultures (Scholze *et al.*, 2002), the effects of nucleotides on depolarization-dependent release were evaluated by determining changes in the ratio of tritium overflow evoked during the two periods of electrical or K⁺ stimulation (S2/S1). When cultures had been subjected to a certain treatment (e.g. cholera toxin or U73122), control experiments were also performed in sister cultures that had not been exposed to that treatment (i.e. remained 'untreated'). In order to directly compare the effects of ADP observed in the absence with those observed in the presence of antagonists, ADP was applied either alone or in combination with the appropriate antagonist. As controls, either the antagonist alone or solvent was applied. Thereafter, the S2/S1 ratio obtained with ADP was expressed as percentage of the corresponding S2/S1 value obtained in its absence (S2/S1, % of control).

Electrophysiology

Currents through Kv7 channels in SCG neurons, so called M currents (I_M), were determined using the perforated patch clamp technique as described previously (Lechner *et al.*, 2003). Currents were recorded at room temperature (20–24°C) from single SCG neurons *in vitro* using an Axopatch 200B amplifier and the pCLAMP 8.0 hardware and software (Molecular Devices, Sunnyvale, CA). Signals were low-pass filtered at 5 kHz, digitized at 10 to 50 kHz and stored on an IBM compatible computer. Traces were analysed off-line by the Clampfit 8.1 programme (Molecular Devices). Patch electrodes were pulled (Flaming-Brown puller, Sutter Instruments, Novato, CA) from borosilicate glass capillaries (Science Products, Frankfurt/Main, Germany), front-filled with a solution consisting of (mM) K₂SO₄ (75), KCl (55), MgCl₂ (8) and HEPES (10), adjusted to pH 7.3 with KOH. Electrodes were then back-filled with the same solution containing 200 µg·mL⁻¹ amphotericin B (in 0.8% DMSO), which yielded tip resistances of 2 to 3 MΩ. The bathing solution contained (mM) NaCl (140), KCl (3.0), CaCl₂ (2.0), MgCl₂ (2.0), glucose (20), HEPES (10), adjusted to pH 7.4 with NaOH. TTX (0.5 µM) was included to suppress voltage-activated Na⁺ currents. ADP and all other drugs were applied via a DAD-12 drug application device (Adams & List, Westbury, NY), which permits a complete exchange of solutions surrounding the cells under investigation within less than 100 ms (Boehm, 1999). To investigate I_M, cells were held at a potential of -30 mV, and three times per minute 1 s hyperpolarizations to -55 mV were applied to deactivate the Kv7 channels; the difference between current amplitudes 20 ms after the onset of hyperpolarizations and 20 ms prior to re-depolarization was taken as a measure for I_M. Amplitudes obtained during the application of test drugs (b) were compared with those measured before (a) and after (c) application of these drugs by calculating $200b / (a + c) = \% \text{ of control}$ or $100 - (200b / [a + c]) = \% \text{ inhibition}$ (Boehm, 1998).

Statistics

Statistical significance of differences between two groups was evaluated by Student's unpaired *t*-tests; when electrophysi-

ological results with nucleotides were obtained in the absence and presence of antagonists in each cell, Student's paired *t*-tests were employed instead (Figure 6). For comparisons between multiple groups, one-way ANOVA followed by Bonferroni's multiple comparison corrections were used. *P*-values < 0.05 were considered as indicating statistical significance.

Materials

(-)-[Ring-2,5,6-³H]-noradrenaline was obtained from PerkinElmer (Vienna, Austria); amphotericin B, ADP, ATP, 2-methylthio-ATP (2-MeSATP), U73122 (1-[6-((17β)-3-methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1*H*-pyrrole-2,5-dione), thapsigargin, H-7 (1-(5-isoquinolinylsulphonyl)-2-methylpiperazine) as well as cholera and pertussis toxin (PTX) were from Sigma; TTX from Latoxan (Rosans, France); MRS 2179 (2'-deoxy-N6-methyladenosine 3',5'-bisphosphate tetrasodium salt) and MRS 2365 ([[(1*R*,2*R*,3*S*,4*R*,5*S*)-4-[6-amino-2-(methylthio)-9*H*-purin-9-yl]-2,3-dihydroxybicyclo [3.1.0]hex-1-yl]methyl] diphosphoric acid mono ester trisodium salt) were from Tocris (Bristol, UK); bulk chemicals were from Merck (Vienna, Austria). Cangrelor was a kind gift from The Medicines Company (Parsippany, NJ). Water-insoluble drugs were first dissolved in DMSO and then diluted into buffer to yield final DMSO concentrations of up to 0.1%, which were also included in control solutions. At these concentrations, DMSO did not affect any of the parameters investigated (Lechner *et al.*, 2003).

Results

Enhancement of stimulation-evoked noradrenaline release from SCG neurons treated with pertussis toxin by ADP

ADP, at a concentration of 100 µM, has been found to reduce noradrenaline release from rat SCG neurons triggered by 30 mM K⁺; however, in neurons treated with pertussis toxin to prevent the signalling via inhibitory G-proteins, the nucleotide tended to enhance stimulation-evoked release (Lechner *et al.*, 2004). Therefore, the effect of ADP was investigated in SCG cultures treated with pertussis toxin (100 ng·mL⁻¹) for 24 h and labelled with [³H]-noradrenaline. In these experiments, 100 µM ADP clearly enhanced tritium overflow triggered by electrical field stimulation, but left spontaneous ³H outflow unaltered (Figure 1A and B). The lack of change in spontaneous outflow suggests that ADP did not trigger action potential-dependent exocytosis, as does the activation of other Gq-coupled receptors, such as B₂ bradykinin receptors (Scholze *et al.*, 2002) or M₁ muscarinic cholinergic receptors (Lechner *et al.*, 2003). To confirm that the effect of ADP was not due to enhanced action potential firing, experiments were repeated in the presence of the Na⁺ channel blocker TTX (0.1 µM). As TTX prevents electrically evoked noradrenaline release from SCG neurons (Boehm, 1999), tritium overflow was stimulated by exposing the cultures to 25 mM KCl for 2 min. Under these conditions, ADP also increased stimulation-evoked tritium overflow (Figure 1C and D) and left the spontaneous outflow unaltered. Thus, the facilitation effect induced by ADP does not require action potential propagation.

The enhancement of stimulation-evoked noradrenaline release from pertussis toxin-treated SCG neurons is mediated by P2Y₁ receptors

Amongst the P2Y receptors, P2Y₁, P2Y₁₂ and P2Y₁₃ are the primary binding sites for ADP (von Kugelgen, 2006). To differentiate between these three, ADP, ATP and 2-MeSATP were chosen as agonists, the latter being a preferred agonist of P2Y₁, but not of P2Y₁₂ and P2Y₁₃, receptors of the rat (von Kugelgen, 2006). All these nucleotides enhanced the electrically evoked ³H overflow from cultures treated with pertussis toxin; from the resulting concentration–response curves, 2-MeSATP was found to be more potent than ADP and ATP, which were about equipotent (Figure 2A). When considering the effects of ATP at concentrations higher than 3 μM, one must not forget the concomitant activation of P2X receptors, which also leads to noradrenaline release from SCG neurons (Boehm, 1999). Nevertheless, the rank order of agonist potency 2-MeSATP > ATP = ADP indicates that P2Y₁ receptors are involved in this effect, even though the concentrations of these nucleotides required to produce this effect were relatively high.

To corroborate the results obtained with the agonistic nucleotides, suramin, reactive blue 2 and MRS 2179 were employed as antagonists and applied together with ADP. While the former two block all three ADP-sensitive P2Y receptors (von Kugelgen, 2006), MRS 2179 is selective for P2Y₁ (Boyer *et al.*, 1998). All three antagonists abolished the facilitation of tritium overflow induced by ADP (Figure 2B),

thereby confirming that this effect was mediated by P2Y₁ receptors.

Enhancement of stimulation-evoked noradrenaline release from SCG neurons not treated with pertussis toxin

The above data indicate that ADP has the ability to enhance stimulation-evoked noradrenaline release when the signalling via inhibitory G proteins is blocked by pertussis toxin. To reveal whether the facilitation by ADP may also occur in neurons with functional Gi/o proteins, experiments were repeated in cultures not treated with PTX; 100 μM ADP caused a significant reduction of electrically-evoked tritium overflow in these cultures (Figure 3A), as described previously where this inhibition of noradrenaline release was suggested to involve P2Y₁₂ receptors (Lechner *et al.*, 2004). Therefore, experiments were performed in the presence of the P2Y₁₂ antagonist cangrelor (Jacobson and Boeynaems, 2010). Cangrelor (10 μM), when applied alone, did not cause obvious changes in tritium outflow (Figure 3C). However, when ADP was applied together with cangrelor, it caused a significant increase in tritium overflow (Figure 3C and D). Hence, the facilitation by ADP can be observed as soon as P2Y₁₂ receptors are blocked.

To reveal whether this facilitatory effect of ADP is mediated by P2Y₁ receptors, the selective and potent P2Y₁ receptor agonist MRS2365 (Chhatriwala *et al.*, 2004) was employed instead of ADP. At a concentration of 0.1 μM, MRS2365 significantly enhanced electrically-evoked tritium overflow

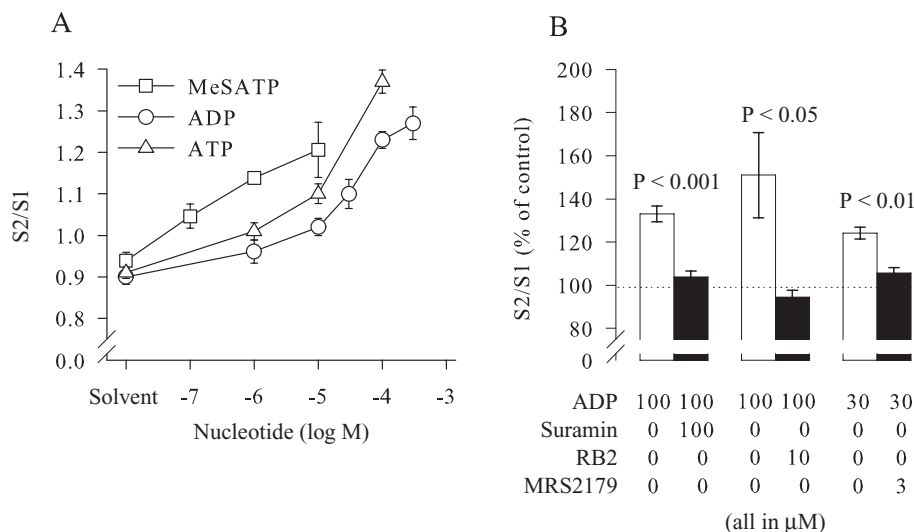


Figure 2

Pharmacological characterization of the receptor mediating the enhancement of [³H]-noradrenaline release. SCG cell cultures were treated with pertussis toxin (PTX; 100 ng·mL⁻¹ for 24 h) and were labelled with [³H]-noradrenaline and superfused. Subsequent to a 60 min washout period, 4 min fractions of superfusate were collected, and tritium overflow was evoked by electrical field stimulation as shown in Figure 1A. (A) The concentration-dependent increase in the S2/S1 ratio of tritium overflow caused by ADP, ATP or 2-MesATP (*n* = 6 to 13). Nucleotides used at the concentrations indicated or the appropriate solvent were present from minute 88 onwards. (B) The increase in the S2/S1 ratio caused by the indicated concentrations of ADP as percentage of control in the absence or presence of the indicated concentrations (in μM) of suramin (*n* = 8–9), reactive blue 2 (RB2; *n* = 10–12) or MRS 2179 (*n* = 8–9). *P*-values for the significance of differences between the results obtained in the absence and presence of antagonists are indicated above the bars.

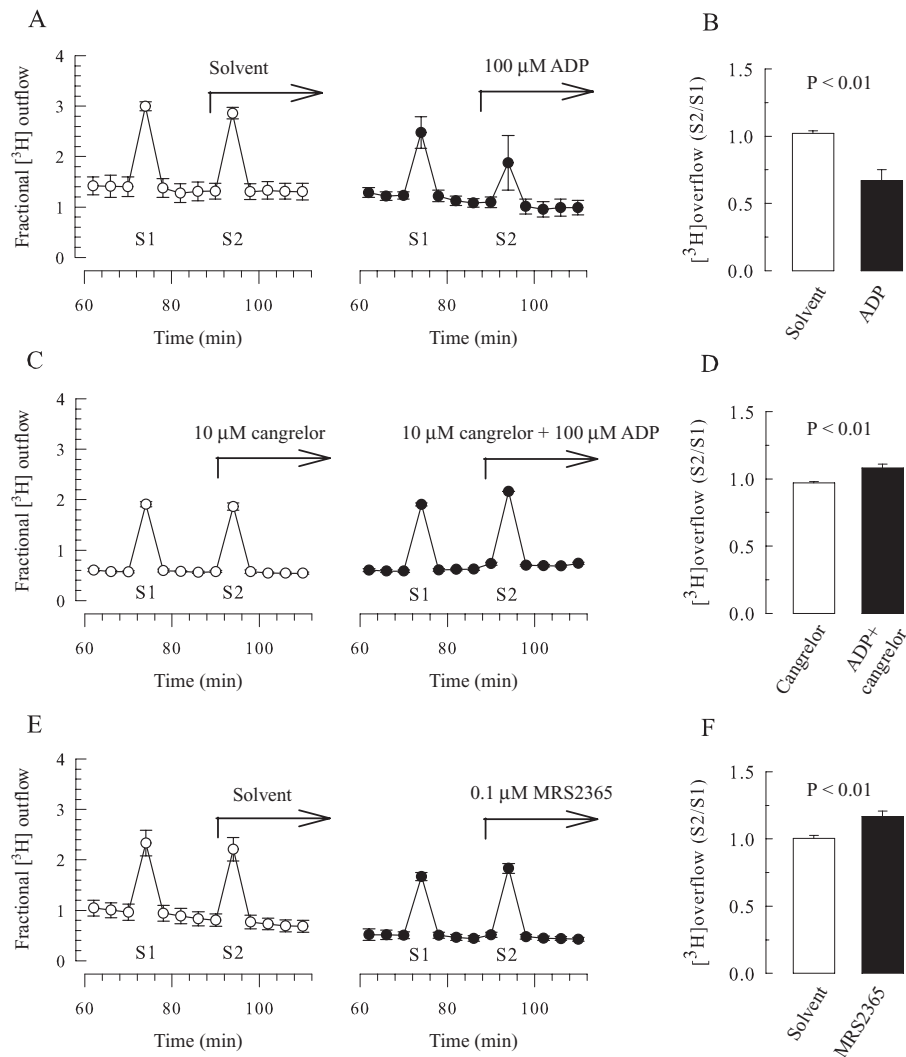


Figure 3

Modulation of [³H]-noradrenaline release from SCG neurons by ADP, MRS 2365 and cangrelor. SCG cell cultures were labelled with [³H]-noradrenaline and superfused. Subsequent to a 60 min washout period, 4 min fractions of superfusate were collected, and tritium overflow was evoked by electrical field stimulation as shown in Figure 1A. (A, C and E) Exemplary time courses of fractional ³H outflow as a percentage of the total radioactivity in the cells ($n = 3$); 100 μM ADP, 10 μM cangrelor, 0.1 μM MRS 2365 or the appropriate solvent were present from minute 88 onwards as indicated by the arrows. (B) S2/S1 ratios obtained in the presence of either solvent or 100 μM ADP ($n = 11$). (D) S2/S1 ratios obtained in the presence of either 10 μM cangrelor or 10 μM cangrelor plus 100 μM ADP ($n = 6$). (F) S2/S1 ratios obtained in the presence of either solvent or 0.1 μM MRS 2365 ($n = 12$); the P -values for the statistical significances of differences (Student's unpaired t -test) are indicated above the bars.

(Figure 3E and F). Thus, the facilitation of tritium overflow can also be seen in cultures not treated with pertussis toxin if either the P2Y₁₂ antagonist cangrelor or the selective P2Y₁ agonist MRS2365 is used. Nevertheless, all future experiments were performed in cultures treated with pertussis toxin to avoid excessive use of these specific P2Y receptor ligands.

The enhancement of stimulation-evoked noradrenaline release from SCG neurons by ADP involves PLC

As the facilitation by ADP was observed in cultures treated with pertussis toxin, this effect cannot be mediated by

inhibitory G-proteins. However, the facilitation of noradrenaline release via presynaptic GPCRs may involve stimulating Gs-proteins (Kubista and Boehm, 2006). To test for this alternative, cultures were treated not only with pertussis toxin, but also with cholera toxin (100 ng·mL⁻¹), both for 24 h; this strategy eliminates α_s G-protein subunits from primary cultures of sympathetic neurons (Boehm *et al.*, 1996). However, the facilitation of electrically evoked ³H overflow was the same in cultures treated with pertussis toxin only as in those treated with pertussis toxin plus cholera toxin (Figure 4A). Thus, the facilitatory effects of ADP do not involve Gs proteins.

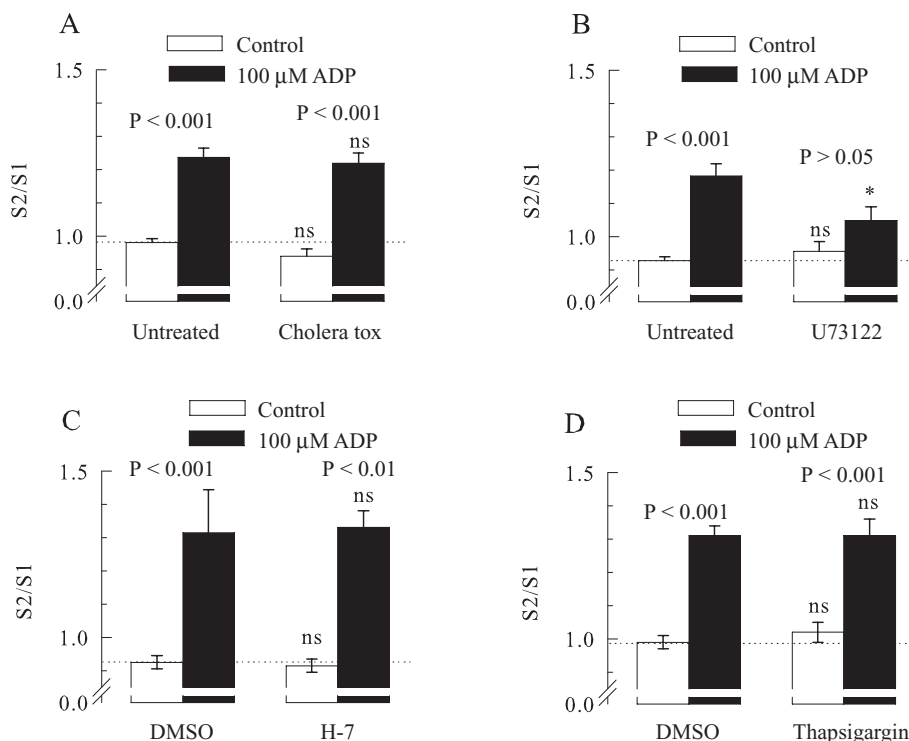


Figure 4

The enhancement of [^3H]-noradrenaline release from rat SCG neurons by ADP involves phospholipase C, but not Gs-proteins, PKC or Ca^{2+} -ATPase. All SCG cell cultures were treated with pertussis toxin (PTX; $100 \text{ ng}\cdot\text{mL}^{-1}$ for 24 h). In addition, some cultures were treated with cholera toxin or remained otherwise untreated. Thereafter, the cultures were labelled with [^3H]-noradrenaline in the absence (untreated) or presence of $3 \mu\text{M}$ U73122 and were then superfused. Subsequent to a 60 min washout period, 4 min fractions of superfusate were collected, tritium overflow was evoked by electrical field stimulation, and ADP was applied as shown in Figure 1A. When appropriate, $10 \mu\text{M}$ H-7, $0.3 \mu\text{M}$ thapsigargin, or 0.1% DMSO were present from minute 50 of superfusion onwards. (A) The S2/S1 ratios of tritium overflow in the absence (control) or presence of $100 \mu\text{M}$ ADP in either untreated or cholera toxin-treated neurons ($n = 10\text{--}12$). (B) The S2/S1 ratios of tritium overflow in the absence or presence of $100 \mu\text{M}$ ADP in either untreated or U73122-treated neurons ($n = 9$). (C) The S2/S1 ratios of tritium overflow in the absence (control) or presence of $100 \mu\text{M}$ ADP applied either in a solution containing DMSO, or in a solution containing H-7 ($n = 9$). (D) The S2/S1 ratios of tritium overflow in the absence (control) or presence of $100 \mu\text{M}$ ADP applied either in a solution containing DMSO or in a solution containing thapsigargin ($n = 9$). P-values for the significance of differences between the results obtained in the absence and presence of ADP are indicated above the bars; * indicates a significant difference versus the corresponding result obtained in untreated cultures at $P < 0.05$; ns indicates no significant difference versus results obtained in either untreated cultures (A and B) or in the presence of DMSO (C and D).

P2Y_1 receptors are most commonly linked to proteins of the Gq family and thereby to PLC (Abbracchio *et al.*, 2006). To test for a role of these latter enzymes, cultures were treated with $3 \mu\text{M}$ U73122, which irreversibly blocks signalling via PLC in SCG neurons (Bofill-Cardona *et al.*, 2000). In neurons treated with U73122 and pertussis toxin, ADP failed to significantly enhance electrically evoked tritium overflow. However, in sister cultures treated with pertussis toxin only, ADP clearly caused facilitation (Figure 4B). Thus, the facilitatory effects of the nucleotide involve activation of PLC.

The enhancement of stimulation-evoked noradrenaline release from SCG neurons does not involve PKC or increases in intracellular Ca^{2+}

Activation of presynaptic receptors linked to PLC can lead to facilitation of noradrenaline release through increases in

intracellular Ca^{2+} and subsequent activation of protein kinase C (Kubista and Boehm, 2006). To test for a role of the latter mechanism, experiments were performed in the presence and absence of $10 \mu\text{M}$ H-7, an inhibitor of PKA, PKC and PKG, with affinities for these enzymes in the low micromolar range (Hidaka *et al.*, 1984). However, the facilitation of electrically-evoked release was the same in the absence and presence of this broad spectrum kinase inhibitor (Figure 4C).

Increases in intracellular Ca^{2+} may facilitate transmitter release independently of protein kinases (Kubista and Boehm, 2006). Therefore, the intracellular Ca^{2+} stores of the SCG neurons were depleted by the Ca^{2+} -ATPase inhibitor thapsigargin (Bofill-Cardona *et al.*, 2000), and the facilitatory effect of ADP was assessed again. However, the facilitation of electrically-evoked ^3H overflow by ADP was the same whether $0.3 \mu\text{M}$ thapsigargin were present or not (Figure 4D). Hence, the effect of ADP is independent of increases in intracellular Ca^{2+} and activation of protein kinases A, C and G.

Recombinant P2Y₁ receptors mediate the enhancement of stimulation-evoked noradrenaline release from PC12 cells treated with pertussis toxin

PC12 cells, in contrast to SCG neurons, do not express endogenous P2Y₁ receptors (Moskvina *et al.*, 2003), but both types of cells do express P2Y₁₂ and P2Y₁₃ receptors (Lechner *et al.*, 2004). To investigate whether P2Y₁ receptors might mediate an enhancement of transmitter release in a neuronal background other than SCG neurons, either non-transfected PC12 cells or a PC12 cell clone stably expressing rat P2Y₁-GFP (Moskvina *et al.*, 2003) were compared with respect to the modulation of stimulation-evoked noradrenaline release by ADP. In agreement with previous results (Lechner *et al.*, 2004), ADP (10 μM) reduced K⁺-evoked tritium overflow from non-transfected PC12 cells by about 50%; however, when these cells had been treated with pertussis toxin (100 ng·mL⁻¹ for 24 h), ADP failed to cause any significant change (Figure 5A). In PC12 cells expressing P2Y₁ receptors, for comparison, 10 μM ADP reduced ³H overflow by only 15% and when the cells had been exposed to pertussis toxin ADP caused a significant enhancement of K⁺-evoked overflow (Figure 5B). Thus, the expression of P2Y₁ receptors in PC12 cells is sufficient to counteract the inhibition of transmitter release induced by ADP and instead ADP enhanced the electrically-evoked release of noradrenaline in the pertussis toxin-treated cells.

P2Y₁ receptors mediate inhibition of Kv7 channels by ADP

Recombinant P2Y₁ receptors mediate inhibition of currents through Kv7 channels (I_M) in PC12 cells (Moskvina *et al.*, 2003). Moreover, ADP and UDP have been found to inhibit I_M in rat SCG neurons, but this effect was suggested to involve P2Y₆ receptors (Boehm, 1998). More recently, evidence has been presented for inhibition of Kv7 channels of SCG

neurons mediated by P2Y₁ receptors (Filippov *et al.*, 2010). Here, we re-evaluated the receptors mediating the inhibition of Kv7 channels by ADP in SCG neurons. In line with previous results, 10 μM ADP reduced I_M relaxation amplitudes, and this effect was entirely reversible (Figure 6A). To determine whether P2Y₁ receptors might mediate this effect, ADP was also applied in the presence of MRS 2179, which abolished the inhibition by ADP (Figure 6A and C). To verify that the antagonism by MRS 2179 (30 μM) was specific for the action of ADP, 10 μM UDP was also used to inhibit I_M; this latter effect, however, remained unaltered in the presence of MRS 2179 (Figure 6D).

To confirm the results obtained with the P2Y₁ antagonist, the selective P2Y₁ agonist MRS 2365 was employed again. As expected, 0.1 μM MRS 2365 also reduced I_M in an entirely reversible manner (Figure 6B), and the inhibition was the same as that induced by 10 μM ADP (Figure 6E). Thus, in rat SCG neurons, P2Y₁ receptors, in addition to P2Y₆, mediate inhibition of Kv7 channels.

Discussion

P2Y₁ receptors are widely distributed in the central and peripheral nervous system and mediate a plethora of effects including the modulation of voltage- and transmitter-gated ion channels (Hussl and Boehm, 2006). However, evidence for presynaptic P2Y₁ receptors is scarce and, if available, only favours inhibitory presynaptic P2Y₁ receptors, as suggested for peripheral sensory neurons (Gerevich *et al.*, 2004), for hippocampal neurons (Rodrigues *et al.*, 2005; Csolle *et al.*, 2008), for spinal cord neurons (Heinrich *et al.*, 2008) and also for sympathetic neurons (Quintas *et al.*, 2009). In general, presynaptic P2Y receptors have been shown to mediate inhibition, but not facilitation, of transmitter release (Goncalves and Queiroz, 2008). Hence, the results of this study provide the first evidence for an

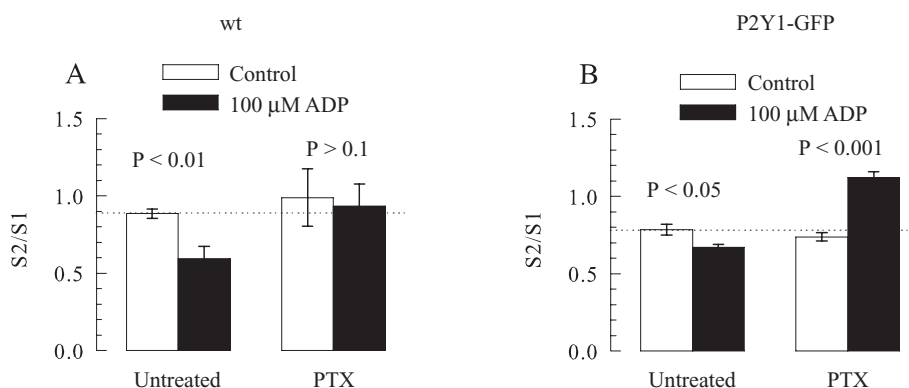


Figure 5

Enhancement of [³H]-noradrenaline release from PC12 cell expressing P2Y₁ receptors by ADP. PC12 cell cultures were treated with pertussis toxin (PTX; 100 ng·mL⁻¹ for 24 h) or remained untreated, were labelled with [³H]-noradrenaline and superfused. Subsequent to a 60 min washout period, 4 min fractions of superfusate were collected. Tritium overflow was stimulated twice (S1 after 72 min and S2 after 92 min of superfusion) by 2 min exposures 25 mM KCl. ADP (10 μM) was applied as shown in Figure 1B. (A) The S2/S1 ratios of tritium overflow from non transfected (wild type; wt) PC12 cells in the absence (control) or presence of 10 μM ADP (*n* = 6). (B) The S2/S1 ratios of tritium overflow from PC12 cells expressing rat P2Y₁-GFP in the absence (control) or presence of 10 μM ADP (*n* = 6). *P*-values for the significance of differences between the results obtained in the absence and presence of ADP are indicated above the bars.

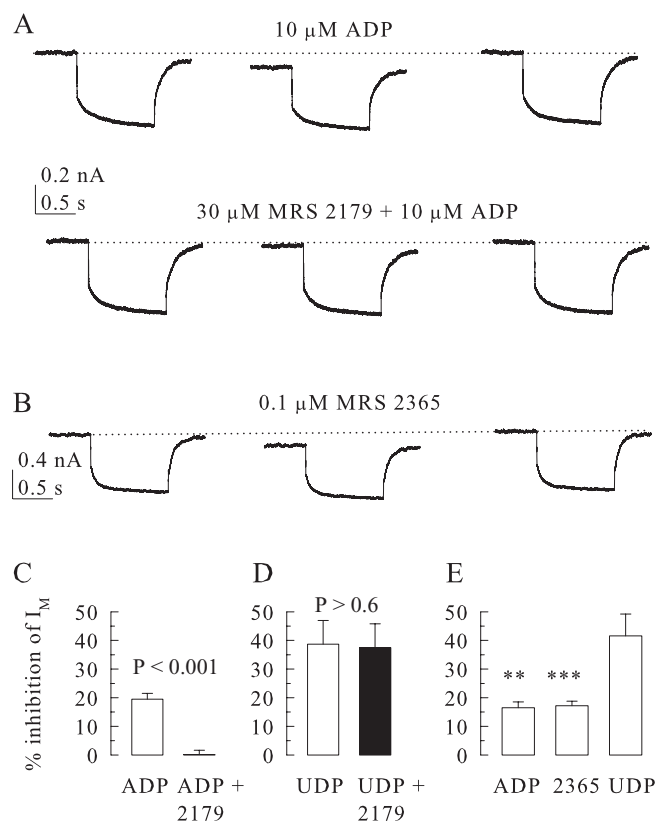


Figure 6

P2Y₁ and P2Y₆ receptors mediate inhibition of currents through Kv7 channels (I_M) in SCG neurons. Currents were activated at -30 mV and quantified by determining the amplitudes of the slow current de-activation relaxations during 1 s hyperpolarizing voltage steps to -55 mV. These I_M amplitudes were determined in the presence of ADP ($10 \mu\text{M}$), UDP ($10 \mu\text{M}$) or MRS2365 ($0.1 \mu\text{M}$) applied either alone or together with MRS2179 ($30 \mu\text{M}$). (A) Original current traces recorded from one neuron before, during and after the exposure to ADP or ADP plus MRS2179. (B) Original current traces recorded from another neuron before, during and after the exposure to MRS2365. (C) Summarizes the inhibition of I_M by ADP in the absence or presence of MRS2179 ($n = 9$). (D) Summarizes the inhibition of I_M by UDP in the absence or presence of MRS2179 ($n = 10$). (E) Summarizes the inhibition of I_M by ADP, UDP or MRS2365 ($n = 6$ to 12). P -values for the significances of differences between the results obtained in the absence and presence of MRS2179 are indicated above the bars; **, *** indicate significant differences versus the inhibition by UDP at $P < 0.01$ and $P < 0.001$, respectively.

enhancement of transmitter release via presynaptic P2Y₁ receptors, thereby providing a demonstration of facilitatory presynaptic P2Y receptors.

In postganglionic sympathetic neurons, multifarious evidence for inhibitory and facilitatory presynaptic P2 receptors has been presented. The pharmacological data support the idea that the nucleotide-dependent presynaptic facilitation involves P2X receptors, whereas the inhibition involves P2Y receptors (Boehm and Kubista, 2002; Sperlagh *et al.*, 2007), most likely P2Y₁₂ (Queiroz *et al.*, 2003; Lechner *et al.*, 2004). This was corroborated in the present study, as ADP caused inhibition of electrically-evoked noradrenaline release from

SCG neurons, which was reversed to facilitation by the P2Y₁₂ antagonist cangrelor (Jacobson and Boeynaems, 2010). Likewise, in SCG neurons treated with pertussis toxin to inactivate the signalling cascades of inhibitory P2Y₁₂ and P2Y₁₃ receptors, ADP also enhanced electrically-evoked noradrenaline release. Moreover, this effect was observed when release was triggered by depolarizing K⁺ concentrations in the presence of TTX in order to block action potential propagation. Thus, the site of action for the facilitation of transmitter release by ADP must be in close proximity to the sites of vesicle exocytosis (i.e. must be a *bona fide* presynaptic receptor).

The presynaptic receptor mediating the facilitation of noradrenaline release was a P2Y receptor, more precisely a P2Y₁ receptor, as indicated by the following results: (i) 2-MesATP, an agonist at rat P2Y₁ receptors (Dixon, 2000) enhanced stimulation-evoked noradrenaline release from SCG neurons treated with pertussis toxin; (ii) 2-MesATP was more potent than ADP as previously demonstrated for rat P2Y₁ receptors (Vohringer *et al.*, 2000); (iii) the facilitation by ADP was abolished by suramin and reactive blue 2, which are both known to be P2Y₁ antagonists (von Kugelgen, 2006); (iv) the selective P2Y₁ antagonist MRS 2179 (Boyer *et al.*, 1998) also abolished the facilitatory affect of ADP; (v) finally, the specific P2Y₁ agonist MRS 2365 (Chhatriwala *et al.*, 2004) enhanced stimulation-evoked noradrenaline release even when the signalling cascades of inhibitory P2Y receptors had not been impaired by pertussis toxin.

The ability of P2Y₁ receptors to mediate facilitation of transmitter release was also confirmed using recombinant receptors. In PC12 cells, the activation of heterologously expressed rat P2Y₁ receptors led to an increase in K⁺-evoked noradrenaline release when signalling via inhibitory GPCRs was prevented by pertussis toxin; this effect was not observed in the absence of P2Y₁ receptors. In PC12 cells expressing P2Y₁ receptors but not treated with the bacterial toxin, ADP caused a reduction of depolarization-evoked noradrenaline release as it did in non-transfected PC12 cells. However, this inhibitory effect of ADP was much more pronounced in non-transfected PC12 cells than in cells expressing P2Y₁ receptors. This indicates that ADP simultaneously activates the facilitatory P2Y₁ receptors and inhibitory P2Y₁₂ or possibly P2Y₁₃ receptors.

P2Y₁ receptors are most commonly linked to phospholipase C via proteins of the Gq family (Abbracchio *et al.*, 2006). In accordance with this notion, the PLC inhibitor U73122 abolished the facilitation by ADP, but removal of Gs proteins by a cholera toxin treatment had no such effect. Activated PLC employs membrane phosphatidylinositol 4,5-bisphosphates to generate inositol trisphosphate and diacylglycerol, which then mediate increases in intracellular Ca²⁺ and activation of PKC, respectively (Suh and Hille, 2007). However, none of these effects was involved in the facilitation of noradrenaline release via presynaptic P2Y₁ receptors, as neither the depletion of intracellular Ca²⁺ stores nor the inhibition of a set of protein kinases including PKC were sufficient to prevent this facilitation.

Heterologously expressed P2Y₁ receptors mediate inhibition of Kv7 channels in SCG neurons (Brown *et al.*, 2000) as well as in PC12 cells (Moskvina *et al.*, 2003). Moreover, ADP has been found to inhibit Kv7 channels in SCG neurons, but it was concluded that this effect is mediated by endogenously

expressed P2Y₆ receptors (Boehm, 1998). Rat SCG neurons are known to express endogenous P2Y₁, P2Y₂, P2Y₄, P2Y₁₂ and P2Y₁₃ in addition to P2Y₆ receptors (Moskvina *et al.*, 2003; Lechner *et al.*, 2004). The present results clearly show that endogenous P2Y₁ receptors contribute to the regulation of Kv7 channels in SCG neurons by nucleotides, as the inhibition by ADP was abolished by the selective P2Y₁ antagonist MRS 2179 (Boyer *et al.*, 1998) and mimicked by the specific P2Y₁ agonist MRS 2365 (Chhatriwala *et al.*, 2004). The inhibition of I_M by UDP, in contrast, was not altered by MRS 2179, thus indicating that P2Y₁ and P2Y₆ receptors can control Kv7 channels independently of each other. While this work was in progress, an inhibition of Kv7 channels in rat SCG neurons by MRS 2365 via endogenous P2Y₁ receptors has been reported by others (Filippov *et al.*, 2010).

Activation of presynaptic Kv7 channels leads to a decrease in transmitter release from cerebrocortical nerve terminals, while inhibition causes the opposite effect (Luisi *et al.*, 2009), and the same holds true for SCG neurons (Hernandez *et al.*, 2008). Moreover, presynaptic muscarinic receptors have been shown to facilitate transmitter release through inhibition of Kv7 channels (Martire *et al.*, 2007). By analogy, the present results suggest that the inhibition of Kv7 channels via P2Y₁ receptors is the basis for the facilitation of noradrenaline release in both PC12 cells and SCG neurons.

In hippocampal neurons, activation of P2Y₁ receptors was found to lead to inhibition of transmitter release (Rodrigues *et al.*, 2005; Heinrich *et al.*, 2008). Although the underlying mechanisms are still obscure, endogenous as well as recombinant P2Y₁ receptors are known to mediate inhibition of neuronal voltage-gated Ca²⁺ channels (Hussl and Boehm, 2006), and this is a prime mechanism of presynaptic inhibition (Brown and Sihra, 2008). Recently, P2Y₁ receptors were also reported to mediate inhibition of Ca²⁺ currents in SCG neurons (Filippov *et al.*, 2010). In the preparation used for this study, ADP also elicits inhibition of voltage-activated Ca²⁺ channels, but this latter effect excludes P2Y₁ and is mediated only by P2Y₁₂ receptors (Kulick and von Kugelgen, 2002; Lechner *et al.*, 2004). The reasons for this discrepancy remain enigmatic, but it is obvious that inhibition of voltage-activated Ca²⁺ channels cannot be the basis for the presynaptic facilitation described here, but rather underlies presynaptic inhibition.

It appears puzzling that one GPCR type like P2Y₁ can mediate presynaptic facilitation or presynaptic inhibition depending on the neuron being investigated. However, this has also been found with other presynaptic Gq-linked receptors, for instance with M₁ muscarinic cholinergic receptors: on the one hand, M₁ receptors mediate an enhancement of noradrenaline release from sympathetic nerve terminals through activation of PKC (Costa *et al.*, 1993; Somogyi *et al.*, 1996); on the other hand, these receptors mediate presynaptic inhibition through the depletion of membrane phosphatidylinositol 4,5-bisphosphate via PLC and the resulting closure of voltage-activated Ca²⁺ channels (Kubista *et al.*, 2009). As mentioned above, P2Y₁ receptors have been reported to inhibit Kv7 channels as well as voltage-activated Ca²⁺ channels in SCG neurons, and the latter effect is determined by the presence or absence of a scaffold protein (Filippov *et al.*, 2010). Hence, depending on the scaffold

proteins nerve terminals are endowed with, one type of GPCR might mediate presynaptic facilitation or inhibition.

In conclusion, the present results demonstrate that ADP controls sympathetic transmitter release not only via inhibitory presynaptic P2Y₁₂ (and maybe P2Y₁₃) receptors but also via facilitatory presynaptic P2Y₁ receptors. The principle that two separate GPCRs for one transmitter family, such as adenine nucleotides, mediate opposing effects at sympathetic nerve terminals is not unknown: noradrenaline and adrenaline activate presynaptic α₂- and β₂-adrenoceptors and thereby cause inhibition and facilitation of noradrenaline release, respectively (Boehm and Kubista, 2002). Since noradrenaline and adenine nucleotides are co-transmitters in postganglionic sympathetic neurons, the pair of inhibitory presynaptic P2Y₁₂ and facilitatory presynaptic P2Y₁ receptors can be viewed as a novel counterpart of the well-established presynaptic adrenoceptors.

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Conflicts of interest

None.

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