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# Inhibition of adenylyl cyclase by neuronal P2Y receptors

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**1** P2Y receptors inhibiting adenylyl cyclase have been found in blood platelets, glioma cells, and endothelial cells. In platelets and glioma cells, these receptors were identified as $P2Y_{12}$. Here, we have used PC12 cells to search for adenylyl cyclase inhibiting P2Y receptors in a neuronal cellular environment.

**2** ADP and ATP (0.1–100 $\mu$M) left basal cyclic AMP accumulation unaltered, but reduced cyclic AMP synthesis stimulated by activation of endogenous $A_{2A}$ or recombinant $\beta_2$ receptors. Forskolin-dependent cyclic AMP production was reduced by $\leqslant 1$ $\mu$M and enhanced by 10–100 $\mu$M ADP; this latter effect was turned into an inhibition when $A_{2A}$ receptors were blocked.

**3** The nucleotide inhibition of cyclic AMP synthesis was not altered when P2X receptors were blocked, but abolished by pertussis toxin.

**4** The rank order of agonist potencies for the reduction of cyclic AMP was ($IC_{50}$ values): 2-methylthio-ADP (0.12 nM) = 2-methylthio-ATP (0.13 nM) > ADP$\beta$S (71 nM) > ATP (164 nM) = ADP (244 nM). The inhibition by ADP was not antagonized by suramin, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid, or adenosine-3′-phosphate-5′-phosphate, but attenuated by reactive blue 2, ATP$\alpha$S, and 2-methylthio-AMP.

**5** RT–PCR demonstrated the expression of $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{12}$, but not $P2Y_1$, receptors in PC12 cells. In Northern blots, only $P2Y_2$ and $P2Y_{12}$ were detectable. Differentiation with NGF did not alter these hybridization signals and left the nucleotide inhibition of adenylyl cyclase unchanged.

**6** We conclude that $P2Y_{12}$ receptors are expressed in neuronal cells and inhibit adenylyl cyclase activity.

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**Keywords:** Adenylyl cyclase; ADP; ATP; neuronal P2Y receptor; PC12; pertussis toxin

**Abbreviations:** A3P5P, adenosine-3′-phosphate-5′-phosphate; ADP$\beta$S, adenosine 5′-O-(2-thiodiphosphate); ATP$\alpha$S, Sp-isomer of adenosine-5′-O-(1-thiotriphosphate); ATP$\gamma$S, adenosine 5′-O-(3-thiotriphosphate); CGS 21680, p-(2-carboxyethyl)phenethylamino-5′-N-ethylcarboxamido-adenosine; NGF, nerve growth factor; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid tetrasodium; RO 20-1724, (3-butoxy-4-methoxybenzyl)imidazoline-2-one; RT–PCR, reverse transcription and polymerase chain reaction; ZM 241385, 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5]triazin-5-ylamino]ethyl]phenol

## Introduction

Adenine and uridine nucleotides exert their actions on either neurons or non-neural cells *via* membrane receptors known as P2 receptors. This family comprises two groups: P2X receptors are ATP-gated ion channels, whereas P2Y receptors belong to the superfamily of G protein-coupled receptors (Ralevic & Burnstock, 1998). At least six DNA sequences coding for putative P2Y receptors have been identified in mammalian species ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$; Ralevic & Burnstock, 1998; Hollopeter *et al*., 2001; Zhang *et al*., 2001). Amongst these, $P2Y_1$ (Tokuyama *et al*., 1995), $P2Y_{11}$ (Communi *et al*., 1997), and $P2Y_{12}$ (Hollopeter *et al*., 2001; Zhang *et al*., 2001) are activated by adenine nucleotides, and $P2Y_6$ by uridine nucleotides (Chang *et al*., 1995). $P2Y_2$ (Lustig *et al*., 1993) and $P2Y_4$ (Communi *et al*., 1995) receptors, in contrast, are sensitive to both adenine and uridine nucleotides. At $P2Y_2$ receptors, UTP and ATP are equipotent agonists (Lustig *et al*., 1993), whereas at P2Y4 receptors, the actions of ATP are species-dependent: ATP is an agonist at rat $P2Y_4$, but an antagonist at human $P2Y_4$ receptors (Kennedy *et al*., 2000). Five of these receptors, namely $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{12}$, have been detected in rats (Ralevic & Burnstock, 1998; Hollopeter *et al*., 2001). In heterologous expression systems, all P2Y receptor subtypes of the rat, with the exception of $P2Y_{12}$, couple to phospholipase C and mediate nucleotide-dependent increases in intracellular inositol phosphates (Ralevic & Burnstock, 1998). Activation of the $P2Y_{12}$ receptor, in contrast, mediates an inhibition of adenylyl cyclase activity (Hollopeter *et al*., 2001). Recombinant rat $P2Y_1$, $P2Y_2$ and $P2Y_6$ receptors also inhibit M-type $K^+$ ($K_M$) channels as well as voltage-gated N-type $Ca^{2+}$ channels, when expressed in neurons (Filippov *et al*., 2000, and references therein).

Aside of cloned P2Y receptors, several native nucleotide receptors have been detected in functional assays and have been characterized by pharmacological means. Most of these receptors were reported to be coupled to phospholipase C and to mediate increases in inositol phosphates. In addition, several reports described P2Y receptors which

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reduced cyclic AMP (Harden *et al*., 1995), and such receptors have been characterized most extensively in human platelets (Daniel *et al*., 1998) and in rat C6 glioma cells (Boyer *et al*., 1993). Most recently, the platelet receptor has been identified as $P2Y_{12}$, and this receptor subtype is also expressed in the glioma cells (Hollopeter *et al*., 2001; Jin *et al*., 2001). An agonist profile similar to that of the $P2Y_{12}$ receptor was reported for a P2Y receptor which also reduced cyclic AMP in a rat brain endothelial cell line (B10), but the authors suggested this receptor to be a $P2Y_1$ subtype (Webb *et al*., 1996). More recently, a detailed pharmacological analysis of the cyclase inhibiting P2Y receptor in B10 endothelial cells indicated that this receptor was not $P2Y_1$, but rather identical to the platelet P2Y receptor which mediates the reduction in cyclic AMP (Simon *et al*., 2001). Taken together, it appears likely that it is $P2Y_{12}$, but not $P2Y_1$ receptors, which mediate adenine nucleotide-dependent inhibition of adenylyl cyclase activity in rat tissues.

In neurons, the activation of endogenous P2Y receptors frequently modulates the functions of voltage-gated ion channels. For instance, in rat sympathetic neurons, uridine nucleotides were found to inhibit M type $K^+$ channels (Boehm, 1998). In rat hippocampal neurons (Dave & Mogul, 1996), ATP has been reported to augment voltage-dependent $Ca^{2+}$ currents, whereas in frog sympathetic neurons (Elmslie, 1992) and in neuroblastoma × glioma hybrid cells (NG108-15; Filippov *et al*., 1996), ATP and other nucleotides diminished $Ca^{2+}$ currents. In the latter cell line, adenine nucleotides were also found to stimulate the synthesis of cyclic AMP (Ohkubo *et al*., 2000). However, neuronal nucleotide receptors that inhibit adenylyl cyclase in order to reduce cyclic AMP have not been described. Here, we used the rat pheochromocytoma cell line PC12 which is ontogenetically related to sympathetic neurons (Greene & Tischler, 1976) to search for such receptors. Our results indicate that these cells express $P2Y_{12}$ receptors which mediate an inhibition of adenylyl cyclase activity.

## Methods

### *Materials*

[2,8-$^3$H]adenine (specific activity 32 Ci mmol$^{-1}$) was obtained from NEN (Vienna, Austria). Na-UTP, Na-UDP, $Na_2$-ATP, Na-ADP, $Li_3$-ADP$\beta$S, Li-GTP, reactive blue 2 (basilene blue E3G), adenosine-3′-phosphate-5′-phosphate (A3P5P), 4-(3-butoxy-4-methoxybenzyl)imidazoline-2-one (RO 20-1724), 3′,5′-cyclic AMP, adenosine 5′-O-(2-thiodiphosphate) (ADP$\beta$S), creatine phosphokinase type III, creatine phosphate, and pertussis toxin were purchased from Sigma (Vienna, Austria); 2-methylthio-AMP, -ADP and -ATP, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid tetrasodium (PPADS), and 2-p-(2-carboxyethyl) phenethylamino-5′-N-ethylcarboxamido-adenosine (CGS 21680) from RBI (Natick, MA, U.S.A.); the Sp-isomer of adenosine-5′-O-(1-thiotriphosphate) (ATP$\alpha$S) from Roche Diagnostics (Vienna, Austria). 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-a][1,3,5] triazin-5-ylamino]ethyl]phenol (ZM 241385) from Tocris Cookson Ltd. (Bristol, U.K.). Suramin was a gift from Bayer, Austria.

### *Cell culture and transfection methods*

PC12 cells were obtained from the European Collection of Cell Cultures (ECACC; Salisbury, U.K.), plated onto collagen-coated (Biomedical Technologies Inc., Stoughton, MA, U.S.A.) 6-well culture dishes (NUNC, Roskilde, Denmark) and were 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, Vienna, Austria), 5% foetal calf serum, and 10% horse serum (both Life Technologies, Vienna, Austria). Once per week, cell cultures were split, and the medium was exchanged twice per week. In order to induce neuronal differentiation, PC12 cells were exposed to recombinant human $\beta$-nerve growth factor (NGF, 50 ng ml$^{-1}$, R&D Systems Inc., Wiesbaden, Germany) for 5–6 days.

Ten $\mu$g of the expression vector pcDNA3 containing the coding sequence for the human $\beta_2$-adrenoceptor (which was kindly provided by M Lohse; see Danner *et al*., 1998) were mixed with 50 $\mu$l of the TransFast transfection reagent (Promega, Mannheim, Germany) and added to one well of semiconfluent PC12 cell cultures in serum-free medium. After 2–3 h, serum was added as described above, and the formation of cyclic AMP was investigated approximately 48 h after this transfection procedure. The transfection efficiency with PC12 cells yielded by this method is typically between 1 and 10%, as determined by the expression of green fluorescent protein.

### *Determination of cyclic AMP*

Semiconfluent PC12 cells were incubated overnight in medium containing 2.5 $\mu$Ci ml$^{-1}$ tritiated adenine in order to label the cellular purines. After 12 h, the medium was replaced by physiological cell buffer (mM): NaCl 120, KCl 3, $MgCl_2$ 2, $CaCl_2$ 2, glucose 20, HEPES 10, adjusted to pH 7.4 with NaOH) containing 100 $\mu$M Ro-20-1724 [4-(3-butoxy-4-methoxybenzyl) imidazolidin-2-one], a phosphodiesterase inhibitor, and 1 u ml$^{-1}$ adenosine deaminase, unless indicated otherwise. In some experiments, $Ca^{2+}$ was omitted from the buffer, and the cultures were washed with buffer twice after removal of the radioactive medium. Dishes were then kept at room temperature for about 90 min. Thereafter, the cells were incubated in buffer containing receptor agonists and/or forskolin for 15 min at room temperature to stimulate adenylyl cyclase. P2 and adenosine receptor antagonists were added 10 min before the addition of respective agonists. The stimulation period was terminated by exchanging the buffer for 1 ml of 2.5% perchloric acid containing 100 $\mu$M non-labelled cyclic AMP followed by a 20 min incubation at 4°C.

Cyclic AMP was separated from the other purines by a two column chromatographic procedure (Johnson *et al*., 1994). One tenth of each sample obtained as described above was used for the determination of the total radioactivity. The remaining 900 $\mu$l were neutralised by addition of 100 $\mu$l 4.2 M KOH and applied to Dowex 50 columns (AG 50W-X4; Bio-Rad, Vienna, Austria) which were then rinsed with 3 ml $H_2O$. The eluate obtained by the subsequent application of 8 ml $H_2O$ was directly poured onto alumina columns (Bio-Rad, Vienna, Austria), which were then washed with 6 ml $H_2O$. Finally, cyclic AMP was eluted with 4 ml imidazole buffer

(20 mM imidazole in 0.2 M NaCl; pH 7.45). Radioactivity within the samples obtained was determined by liquid scintillation counting.

### *Calculations and statistics*

The radioactivity retrieved within the fraction of cyclic AMP was expressed as a percentage of the total radioactivity incorporated in the cells. Stimulation of the cells with adenosine receptor agonists or forskolin consistently caused a multifold increase in these values of cyclic AMP (see, for instance, Figures 1A and 2B), but the extent of basal and stimulated cyclic AMP synthesis varied between different preparations. Therefore, the values of cyclic AMP obtained in the presence of various agonists and/or forskolin were normalized to the values obtained in their absence within the same preparation (normalized to basal).

In order to compare the modulation of stimulated cyclic AMP accumulation by, for instance, nucleotides, values obtained in the presence of modulators were expressed as percentage of the values obtained in their absence and in the presence of stimulators, such as forskolin or CGS 21680, only (per cent of control). Accordingly, the extent of inhibition of stimulated cyclic AMP accumulation by various nucleotides was calculated as per cent inhibition = 100 − (per cent of control).

All values represent arithmetic means ± standard errors of the mean; $n$ values represent the number of culture wells investigated. Significances of differences between single data points were analysed by the Mann-Whitney test. Concentration-response curves were fitted to experimentally obtained data by the ALLFIT programme (DeLean *et al*., 1978) which provides estimates (± s.e.m.) for half maximal concentrations as well as minimal and maximal effects and determines the qualities of fitted results. Differences between single concentration-response curves were determined by simultaneous fitting with shared parameters and subsequent calculation of the F-statistic on the resulting 'extra sum of squares' (DeLean *et al*., 1978).

### *RNA Isolation, RT–PCR, and Northern blots*

The methods of P2Y receptor detection by RNA isolation and subsequent Northern blot or PCR analysis have been described previously for primary cultures of sympathetic neurons (Vartian *et al*., 2001). Here, total RNA was extracted from PC12 cell cultures using the peqGOLD RNAPure Reagent (PeqLab, Erlangen, Germany). First strand cDNA was synthesised with Superscript reverse transcriptase (Life Technologies, Vienna, Austria) according to the manufacturer's instructions. 5 $\mu$g of total RNA were mixed with 0.5 $\mu$l oligo-dT (0.5 $\mu$g $\mu$l$^{-1}$; Promega), 1 $\mu$l of 10 mM dNTPs, incubated at 65°C for 5 min and quickly chilled on ice. Reverse transcription buffer, dithiothreitol, and 0.5 $\mu$l of the ribonuclease inhibitor RNAsin (80 u $\mu$l$^{-1}$; Promega) were added, and the mixture was incubated at 42°C for 2 min prior to the addition of the Superscript (Life Technologies, Vienna, Austria) enzyme. Where indicated, reverse transcriptase was omitted to exclude a possible contamination by genomic DNA.

One $\mu$l of cDNA was used for PCR amplification with Taq DNA polymerase (Roche Diagnostics, Vienna, Austria). Specific primers and cycling conditions described by Harper *et al*. (1998) were used to amplify fragments of $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors and those described by Arslan *et al*. (2000) to amplify a fragment of $P2Y_6$. Specific primers were designed to amplify a 524 bp fragment of the rat $P2Y_{12}$ receptor sequence: Sense primer-ctttggcaacgaaaccaagt, and antisense primer-atggtctggtggtcttcagg. PCR was initiated by a 3 min denaturation at 94°C, followed by 40 cycles of 30 s at

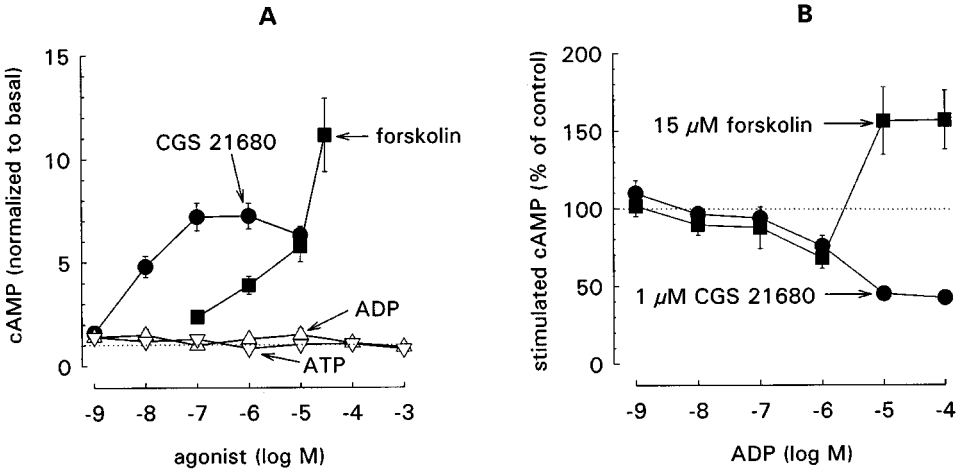


**Figure 1** Regulation of cyclic AMP synthesis in PC12 cells by forskolin and agonists at P1 and P2 receptors. After loading with [$^3$H]-adenine, PC12 cells were incubated in a phosphodiesterase inhibitor (Ro 20-1724, 100 $\mu$M) for 105 min. During the last 15 min of this incubation period, the indicated concentrations of forskolin, CGS 21680, ADP and ATP, respectively, were also present. (A) PC12 cells were exposed to forskolin (0.1 to 30 $\mu$M; $n = 5$), CGS 21680 (1 nM to 10 $\mu$M; $n = 9$), ADP or ATP (1 nM to 1 mM; $n = 6$ for each nucleotide) for 15 min. The amount of radioactivity retrieved within the fraction of cyclic AMP (cAMP) was calculated as percentage of the total radioactivity in the cell culture. Thereafter, the values obtained in the presence of these drugs were normalized to the data obtained in their absence (normalized to basal). (B) PC12 cells were exposed to either 15 $\mu$M forskolin ($n = 9$) or 1 $\mu$M CGS 21680 ($n = 6$) together with the indicated concentrations of ADP. Results obtained in the presence of ADP are expressed as percentage of those obtained in its absence (per cent of control; see Methods).

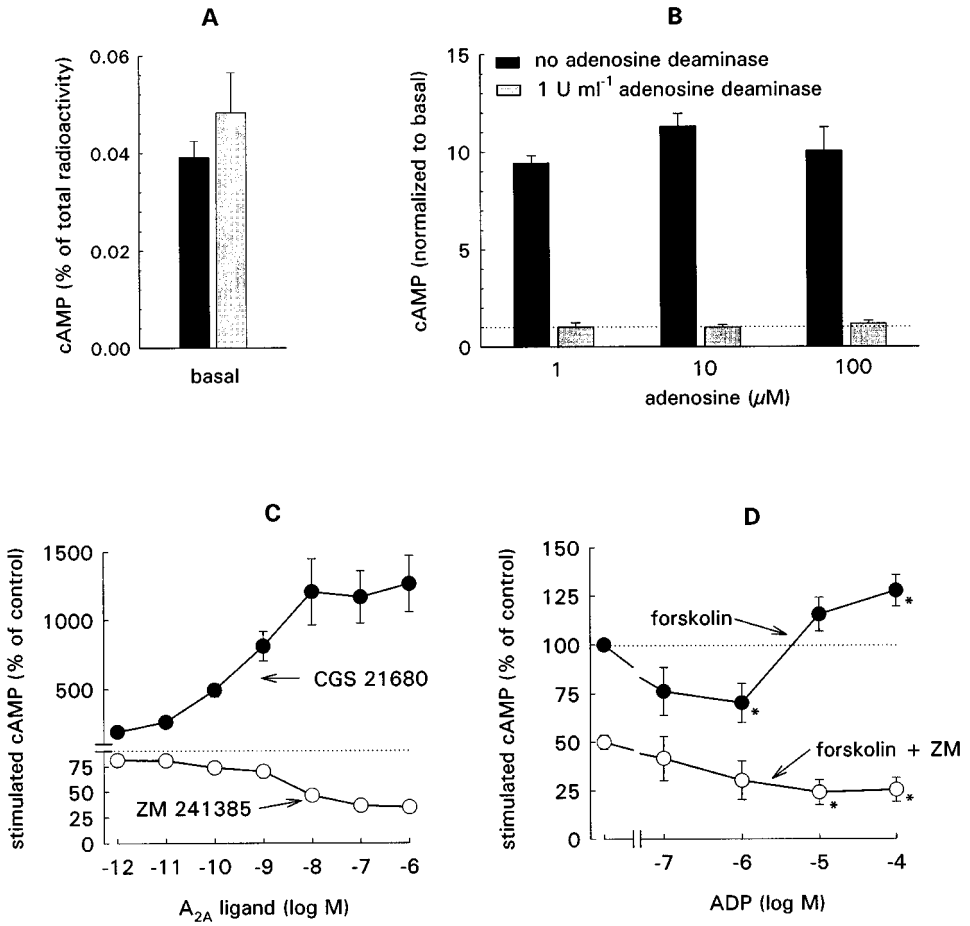


**Figure 2** Enhancement of forskolin-dependent cyclic AMP accumulation by ADP *via* $A_{2A}$ receptors. After loading with [$^3$H]-adenine, PC12 cells were incubated in RO 20-1724 (100 $\mu$M for 105 min). During the last 15 min of this incubation period, 15 $\mu$M forskolin and/or the indicated concentrations of receptor ligands were also present. (A) PC12 cells were exposed to RO 20-1724 for 105 min in the absence or presence of adenosine deaminase (1 u ml$^{-1}$). The amount of radioactivity retrieved within the fraction of cyclic AMP (cAMP) was calculated as percentage of the total radioactivity in the cell culture; n = 6. (B) PC12 cells were exposed to the indicated concentrations of adenosine for 15 min in the absence or presence of adenosine deaminase (1 u ml$^{-1}$). Values obtained in the presence of adenosine were normalized to the data obtained in its absence (normalized to basal); $n = 6$. Note that adenosine deaminase (1 u ml$^{-1}$) was present in all other experiments. (C) PC12 cells were exposed for 15 min to 15 $\mu$M forskolin together with the indicated concentrations of either CGS 21680 ($n = 6$ to 9) or ZM 241385 ($n =$ 6 to 9). Results obtained in the presence of these $A_{2A}$ receptor ligands are expressed as percentage of those obtained in their absence (per cent of control; see Methods). (D) PC12 cells were exposed for 15 min to 15 $\mu$M forskolin plus the indicated concentrations of ADP together with ($n = 6$), or without ($n = 9$), 1 $\mu$M ZM 241385. Results obtained in the presence of ADP and/or ZM 241385 are expressed as percentage of those obtained in their absence (per cent of control; see Methods). *Indicates significances of differences at $P < 0.05$ between the results obtained in the absence and presence of ADP, respectively.

94°C, 30 s at 60°C and 30 s at 72°C, and terminated by a final extension for 7 min at 72°C. After amplification, the PCR fragments were separated by electrophoresis through 2% agarose gels, isolated, and subsequently cloned into the pCR3.1 vector (Invitrogen). Plasmid DNA was isolated from positive clones using the Plasmid Midi Kit (Qiagen, Hilden, Germany) and sequenced in order to verify the P2Y receptor identity.

For Northern analysis, 20 $\mu$g RNA per lane were separated by gel electrophoresis through formaldehyde containing 1.5% agarose gels and transferred to nylon membranes. After UV-cross-linkage, membranes were hybridized overnight at 65°C in a hybridization solution which contained (mM): Pipes (pH 6.5) 50, NaCl 100, sodium phosphate buffer (pH 7.0) 50, EDTA (pH 8.0) 1 and 5% sodium dodecyl sulphate (SDS). After washing, the blots were exposed to X-ray films and subsequently the probes were removed from the membranes which were resubjected to further hybridization with additional P2Y receptor-specific probes. *Eco*RI fragments carrying the above P2Y receptor sequences were labelled with [$\alpha$-$^{32}$P]deoxycytidine triphosphate by random priming using the Prime-a-Gene labelling system (Promega) and were used as subtype-specific probes (Vartian *et al.*, 2001).

## Results

After labelling of cellular purines of PC12 cells with [$^3$H]-adenine, 591.2 ± 52.7 c.p.m. ($n = 27$) were retrieved within the fraction of cyclic AMP, which corresponded to 0.054 ± 0.004% of the total radioactivity in the cultures. As these values were obtained after incubation of PC12 cells in

the phosphodiesterase inhibitor RO 20-1724 for 105 min, changes in these basal values of cyclic AMP will reflect changes in adenylyl cyclase activity rather than alterations in cyclic AMP degradation. In all subsequent experiments, results obtained in the presence of receptor agonists and/or forskolin were normalized to these basal values of cyclic AMP accumulation obtained in the absence of these drugs.

### *ADP modulates stimulation-dependent, but not spontaneous, cyclic AMP accumulation*

Exposure of PC12 cells to either the $A_{2A}$ adenosine receptor agonist CGS 21680 or to the direct adenylyl cyclase activator forskolin for 15 min caused concentration-dependent increases in cellular cyclic AMP. In contrast, exposure of the cells to ADP or ATP, both at 1 nM to 100 $\mu$M, for the same period of time left the basal levels of cyclic AMP unchanged (Figure 1A). However, when ADP was applied together with 1 $\mu$M CGS 21680, the nucleotide caused a concentration-dependent reduction of cyclic AMP formed in response to $A_{2A}$ receptor activation. When ADP was applied together with 15 $\mu$M forskolin, the purine nucleotide reduced cyclic AMP at concentrations $\leqslant 1$ $\mu$M, but enhanced the forskolin-dependent cyclic AMP synthesis at 10 and 100 $\mu$M (Figure 1B).

### *ADP augments forskolin-dependent cyclic AMP accumulation via $A_{2A}$ adenosine receptors*

The biphasic effect of ADP on forskolin-stimulated cyclic AMP accumulation was unexpected, and several types of experiments aimed at identifying the underlying mechanisms. Adenine nucleotides are rapidly degraded by a cascade of nucleotidases to generate adenosine as the final product (Zimmermann, 2000). We therefore investigated whether the nucleoside might be involved in the enhancing action of ADP. To this end, adenosine itself was applied in the absence and presence of adenosine deaminase (1 u $ml^{-1}$), which has been included in all other experiments. Basal cyclic AMP accumulation was not altered by the omission of adenosine deaminase (Figure 2A). However, adenosine at 1 to 100 $\mu$M markedly stimulated cyclic AMP synthesis in the absence of this enzyme, but left cyclic AMP unaltered in its presence (Figure 2B). Thus, in the presence of adenosine deaminase, i.e. under our routine experimental conditions, adenosine is unable to stimulate cyclic AMP formation. Therefore, the nucleoside is unlikely to contribute to any of the effects of adenine nucleotides.

Alternatively, ADP might modulate the cyclic AMP accumulation triggered by forskolin through a direct action on $A_{2A}$ receptors. In fact, $A_{2A}$ receptor activation has been shown to contribute to the accumulation of cyclic AMP in PC12 cells when exposed to forskolin (Florio *et al*., 1999a). Accordingly, the $A_{2A}$ receptor antagonist ZM 241385 reduced the amount of cyclic AMP produced in the presence of forskolin in a concentration-dependent manner. Furthermore, the $A_{2A}$ receptor agonist CGS 21680 potentiated the stimulatory effect of forskolin on cyclic AMP (Figure 2C) and appeared to be at least 10 fold more potent in the presence of forskolin as compared to its absence (compare Figures 1A and 2C). Finally, in the presence of the $A_{2A}$ antagonist ZM 241385 (1 $\mu$M), ADP caused only inhibition, but not augmentation, of the cyclic AMP accumulation elicited by forskolin, even though in sister cultures not treated with ZM 241385 the enhancing effect was observed at 10–100 $\mu$M ADP (Figure 2D).

The above results indicate that the enhancing action of ADP was related to activation of $A_{2A}$ receptors which appear to be functionally sensitized in the presence of forskolin. To corroborate that this effect was specific for the forskolin stimulation of adenylyl cyclase, PC12 cells were transiently transfected with human $\beta_2$-adrenoceptors and subsequently adenylyl cyclase was activated by 10 $\mu$M isoprenaline. This $\beta$-adrenergic agonist caused a $2.99 \pm 0.41$ fold increase in cyclic AMP synthesis ($n=10$), which was reduced down to a $1.24 \pm 0.17$ fold increase in the presence of 10 $\mu$M ADP ($n=10$; $p<0.001$ vs isoprenaline alone). Thus, the enhancing effect of ADP was specific for the stimulation of adenylyl cyclase activity by forskolin. In all subsequent experiments, the inhibitory effects of nucleotides on stimulated cyclic AMP synthesis were investigated only together with the $A_{2A}$ receptor agonist CGS 21680 (1 $\mu$M) as adenylyl cyclase stimulating agent.

### *The inhibition of adenylyl cyclase by nucleotides involves P2Y, but not P2X, receptors*

To obtain preliminary information on the receptor involved in the inhibition of adenylyl cyclase activity by ADP, the action of this nucleotide was compared with those of UDP, UTP, and ATP. At 1 to 10 $\mu$M, only the adenine, but not the uridine nucleotides, inhibited the CGS 21680-induced accumulation of cyclic AMP (Figure 3A). The uridine nucleotides also failed to alter basal cyclic AMP synthesis (not shown).

Within the family of P2 receptors, $P2Y_1$, $P2Y_{11}$, and $P2Y_{12}$ as well as all P2X receptors are sensitive to adenine, but not to uridine, nucleotides. Furthermore, only P2Y, but not P2X receptors, are activated by both ADP and ATP. To reveal whether the effect of ATP might also involve activation of a ligand-gated cation channel, i.e. of a P2X receptor, experiments were performed in the absence of extracellular $Ca^{2+}$ and the results were compared with those obtained in its presence. The rationale underlying this experiment is the high $Ca^{2+}$-permeability of P2X receptors (Ralevic & Burnstock, 1998) and a possible inhibition of adenylyl cyclase activity through increases in intracellular $Ca^{2+}$ (Simonds, 1999). Basal accumulation of cyclic AMP was slightly higher in the presence of 2 mM $Ca^{2+}$ ($0.042 \pm 0.006$% of total radioactivity; $n=6$) than in its absence ($0.029 \pm 0.004$% of total radioactivity; $n=6$; $P<0.05$), but the CGS 21680-dependent cyclic AMP production was not affected by the absence of this cation (not shown). Furthermore, in the absence of extracellular $Ca^{2+}$, 10 and 100 $\mu$M ATP, concentrations that suffice to activate P2X receptors in PC 12 cells (Vartian & Boehm, 2001), still reduced cyclic AMP accumulation, and the effects in the presence and absence of extracellular $Ca^{2+}$ were not significantly different from each other (Figure 3B).

The above results indicate that $Ca^{2+}$ entry *via* P2X receptors does not contribute to the inhibitory effects of ATP. To verify that other signalling mechanisms downstream of P2X receptor activation are also not involved in the actions of ATP, 100 $\mu$M of the nucleotide was tested in the

presence of 100 $\mu$M suramin and PPADS, respectively. These antagonist concentrations entirely prevent P2X receptor activation in PC12 cells (Vartian & Boehm, 2001). Nevertheless, these drugs failed to attenuate the inhibition of adenylyl cyclase activity by 100 $\mu$M ATP, but rather enhanced the inhibitory effect of the nucleotide (Figure 3C).

In most cases, receptor-dependent inhibition of adenylyl cyclase activity involves pertussis toxin-sensitive G proteins (Simonds, 1999). To finally prove that the reduction of cyclic AMP accumulation was mediated by a G protein-coupled, i.e. by a P2Y, receptor, cultures were treated with this toxin (100 ng ml$^{-1}$ for 24 h), and the effect of ADP was reinvestigated. In the pretreated cultures, ADP failed to significantly alter the CGS 21680-induced accumulation of cyclic AMP (Figure 3D). In untreated sister cultures, however, ADP caused a concentration-dependent inhibition as also shown in Figure 1B.

### *2-methylthio-ADP and -ATP are the most potent agonists inhibiting adenylyl cyclase activity in PC12 cells*

In order to characterize the receptor mediating the nucleotide-dependent inhibition of adenylyl cyclase in more detail, ATP, ADP, ADP$\beta$S, and 2-methylthio-ADP and -ATP were

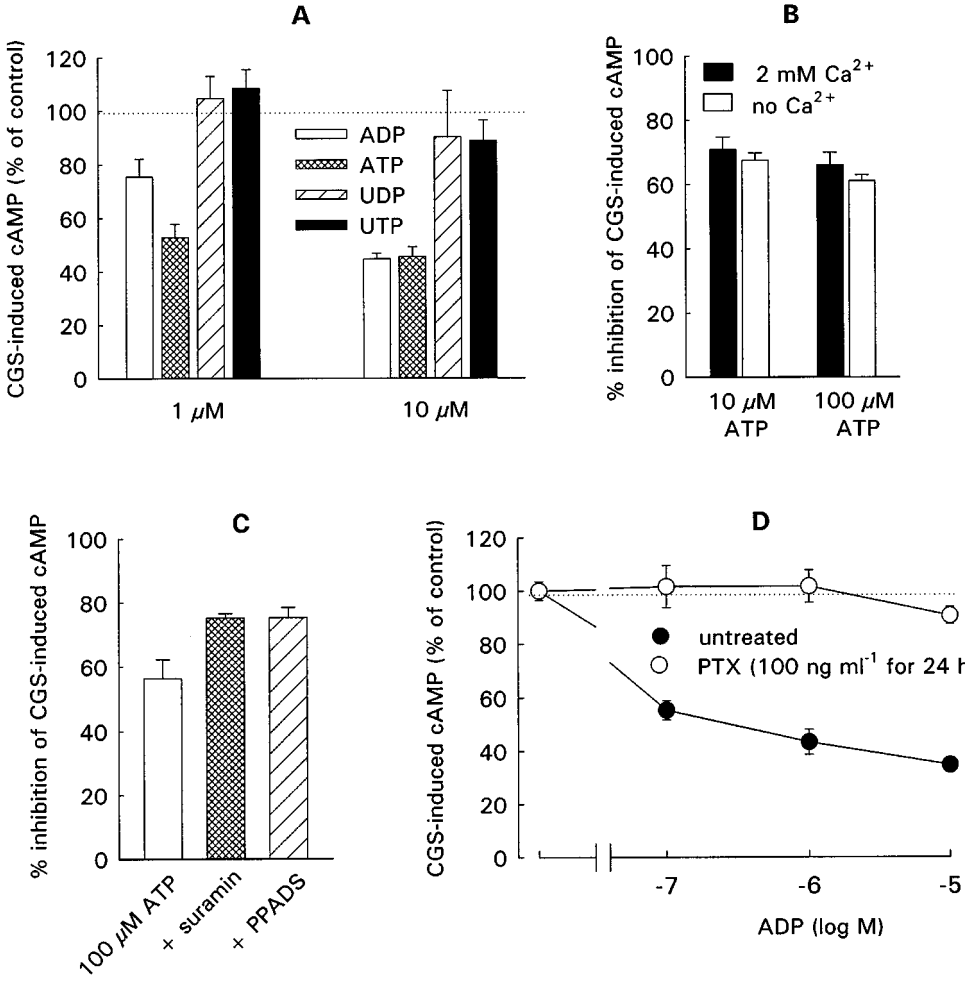


**Figure 3** Inhibition of $A_{2A}$ receptor-dependent cyclic AMP accumulation by adenine nucleotides *via* P2Y, but not via P2X, receptors. After loading with [$^3$H]adenine, PC12 cells were incubated in RO 20-1724 (100 $\mu$M) for 105 min. During the last 15 min of this incubation period, 1 $\mu$M CGS 21680 and the indicated concentrations of P2 receptor ligands were also present. The amount of radioactivity retrieved within the fraction of cyclic AMP (cAMP) was calculated as percentage of the total radioactivity in the cell culture, and values obtained in the presence of CGS 21680 were normalized to the data obtained in its absence (normalized to basal). (A) CGS 21680 was applied alone or together with ADP ($n=12$), ATP ($n=9$), UDP ($n=6$), and UTP ($n=9$), respectively. Results obtained in the presence of these nucleotides are expressed as percentage of those obtained in their absence (per cent of control; see Methods). (B) All incubations were performed in the presence or absence of extracellular $Ca^{2+}$ (2 mM; $n=6$ in both cases). CGS 21680 was applied alone or together with 10 or 100 $\mu$M ATP, respectively. The differences between the results obtained in the presence of ATP and those obtained in its absence are shown as per cent inhibition by ATP (see Methods). (C) CGS 21680 was applied alone or together with 100 $\mu$M ATP, 100 $\mu$M suramin and/or 100 $\mu$M PPADS, respectively. The differences between the results obtained in the presence of ATP and those obtained in its absence are shown as % inhibition by ATP (see Methods). (D) In one half of the culture dishes, PC12 cells were treated with pertussis toxin (100 ng ml$^{-1}$) for 24 h prior to the experiments. CGS 21680 was applied alone or together with 0.1 to 10 $\mu$M ADP ($n=9$ in each case). Results obtained in the presence of ADP are expressed as percentage of those obtained in its absence (per cent of control; see Methods).

applied at concentrations of 0.01 nM to 1 mM (Figure 4A). The inhibition of CGS 21680-stimulated cyclic AMP accumulation by ADP was half maximal at $244 \pm 92$ nM and the maximum inhibition by ADP amounted to $39.5 \pm 2.1\%$ of control. ATP was equipotent to ADP in reducing the $A_{2A}$ receptor-dependent adenylyl cyclase activity and achieved half maximal effects at $164 \pm 68$ nM. ADP$\beta$S was somewhat more potent than ADP and caused half-maximal reduction of cyclic AMP accumulation at $71 \pm 24$ nM ($P<0.05$ vs ADP). The most potent agonists were 2-methylthio-ADP and -ATP which elicited half maximal inhibition at $0.12 \pm 0.04$ and $0.13 \pm 0.06$ nM, respectively. Maximally effective concentrations of all of the agonists applied reduced the CGS 21680-induced accumulation of cyclic AMP to the same extent as ADP ($P>0.05$ vs ADP in each case).

ATP preparations may contain amounts of ADP sufficient to activate P2Y receptors (Hechler *et al*., 1998b). To find out whether the actions of ATP were caused by diphosphate contaminations, ATP containing solutions were treated with an ATP regenerating system provided by creatine phosphokinase (20 u ml$^{-1}$) and 10 mM creatine phosphate for at least 90 min, which has been shown to virtually eliminate diphosphate contaminations (Hechler *et al*., 1998b). In addition, in PC12 cell cultures, ADP may be generated from ATP by ectonucleotidases (Vollmayer *et al*., 2001). For this reason, 1 $\mu$M ATP was applied to PC12 cells together with creatine phosphokinase (20 u ml$^{-1}$) and 10 mM creatine phosphate. This procedure did not significantly alter the inhibition of CGS 21680-induced cyclic AMP accumulation by ATP ($36.5 \pm 7.7\%$ of control in the absence of creatine phosphokinase and creatine phosphate and $38.2 \pm 2.8\%$ of control in its absence; $n=6$; $P>0.1$). Thus, ATP itself, and not the degradation product ADP, inhibited the accumulation of cyclic AMP.

To further characterize the receptor involved, ADP (10 $\mu$M) was applied together with three typical P2 antagonists, namely PPADS (100 $\mu$M), suramin (100 $\mu$M) and reactive blue 2 (30 $\mu$M). Amongst these, only reactive blue 2 attenuated the inhibitory action of the nucleotide. Suramin, in contrast, had no effect at all, and PPADS enhanced rather than reduced the inhibition by ADP (Figure 4B).

### *The receptor mediating the inhibition of adenylyl cyclase by nucleotides resembles P2Y$_{12}$, but not P2Y$_1$, receptors*

The data presented above indicate that a P2Y receptor subtype exerts inhibitory control over adenylyl cyclase activity in PC12 cells. The lack of effect of uridine nucleotides and the high potencies of the 2-methylthio adenine nucleotides suggest a role of either P2Y$_1$- or P2Y$_{12}$-like receptors. To distinguish between these two, several additional P2Y receptor antagonists were tested. ADP (10 $\mu$M) was applied in presence of 100 $\mu$M of the selective P2Y$_1$ receptor antagonist A3P5P (Boyer *et al*., 1996) which, however, failed to alter the inhibitory action of the nucleotide (Figure 4C). In contrast, ATP$\alpha$S (100 $\mu$M) which does not block (Hechler *et al*., 1998a), but rather activates (Vöhringer *et al*., 2000), P2Y$_1$ receptors clearly attenuated the inhibition by ADP. Finally, 2-methylthio-AMP which blocks recombinant P2Y$_{12}$ receptors (Hollopeter *et al*., 2001) significantly reduced the inhibitory effect of ADP when applied at 10 and 100 $\mu$M.

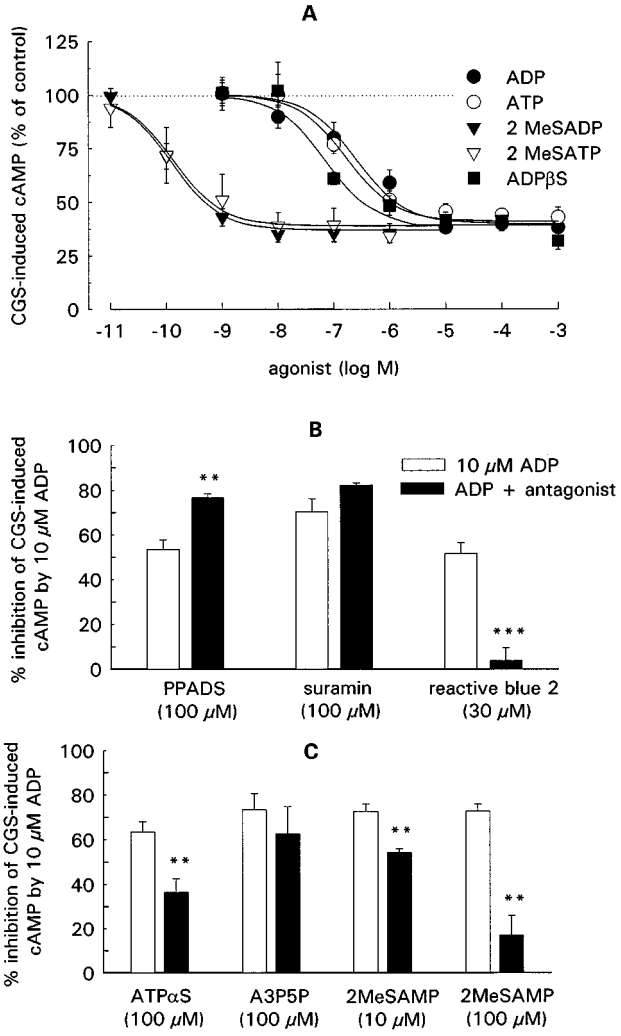


**Figure 4** Pharmacological characteristics of the P2Y receptor mediating the inhibition of $A_{2A}$ receptor-dependent cyclic AMP accumulation. After loading with [$^3$H]-adenine, PC12 cells were incubated in RO 20-1724 (100 $\mu$M) for 105 min. During the last 15 min of this incubation period, 1 $\mu$M CGS 21680 and the indicated concentrations of P2 receptor ligands were also present. The amount of radioactivity retrieved within the fraction of cyclic AMP (cAMP) was calculated as percentage of the total radioactivity in the cell culture, and values obtained in the presence of CGS 21680 were normalized to the data obtained in its absence (normalized to basal). (A) CGS 21680 was applied alone or together with 2-methylthio-ADP ($n=9$), 2-methylthio-ATP ($n=9$), ADP$\beta$S ($n=9$), ADP ($n=9-12$), and ATP ($n=9$), respectively. Results obtained in the presence of these nucleotides are expressed as percentage of those obtained in their absence (% of control; see Methods). (B) CGS 21680 was applied alone or together with 10 $\mu$M ADP. Where indicated, 100 $\mu$M PPADS, 100 $\mu$M suramin, and 30 $\mu$M reactive blue 2, respectively, were also present. The differences between the results obtained in the presence of ADP with or without antagonists and those obtained in its absence are shown as per cent inhibition by ADP (see Methods). **, *** indicate significances of differences between the results obtained in the absence and presence of antagonists at $P<0.01$ and $P<0.001$, respectively. (C) CGS 21680 was applied alone or together with 10 $\mu$M ADP. Where indicated, 100 $\mu$M ATP$\alpha$S, 100 $\mu$M A3P5P, and 10 or 100 $\mu$M 2-methylthio-AMP, respectively, were also present. The differences between the results obtained in the presence of ADP with or without antagonists and those obtained in its absence are shown as per cent inhibition by ADP (see Methods). **Indicates significances of differences between the results obtained in the absence and presence of antagonists at $P<0.01$.

### *PC12 cells express $P2Y_{12}$, but not $P2Y_1$, receptors*

In order to correlate the pharmacological results presented above with molecularly defined P2Y receptor subtypes of the rat, we investigated the P2Y receptor expression pattern of two different PC12 cell clones by RNA extraction followed by reverse transcription and PCR analysis with primers predicted to recognize DNA coding for all known rat P2Y receptor subtypes. Separation of the PCR products by agarose gel electrophoresis revealed bands of approximately 350 to 500 bp lengths, respectively, after amplification with $P2Y_2$, $P2Y_4$, $P2Y_6$ and $P2Y_{12}$-specific primers, but no band after amplification with a $P2Y_1$-specific primer (Figure 5A). These four bands were absent when the reverse transcriptase had been omitted from the reverse transcription step and were thus not due to the presence of contaminating genomic DNA. DNA sequencing showed that these bands corresponded to fragments of rat $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{12}$ receptors. Thus, PC12 cells do express $P2Y_{12}$, but not $P2Y_1$, receptors.

### *Neuronal differentiation of PC12 cells does not alter P2Y receptor expression and function*

Exposure of PC12 cells to nerve growth factor (NGF) induces the development of a neuronal phenotype. In parallel, the expression of several neurotransmitter receptors is altered, and $A_{2A}$ receptors, for instance, have been reported to be downregulated by NGF (Arslan *et al*., 1997), whereas P2Y receptors were shown to be upregulated (Arslan *et al*., 2000). In order to find out whether neuronal differentiation might alter the function of the adenylyl cyclase inhibiting P2Y receptor, PC12 cells were exposed to 50 ng ml$^{-1}$ NGF for 5 days, and the reduction of cyclic AMP formation by ADP and ATP was investigated afterwards. The NGF treatment did not affect basal cyclic AMP accumulation, but reduced the cyclic AMP synthesis triggered by 1 $\mu$M CGS 21680 by $19.2 \pm 5.5\%$ ($P < 0.05$) which corroborates the downregulation of $A_{2A}$ receptors (Arslan *et al*., 1997). However, the inhibitory actions of ADP and ATP remained largely unaffected (Figure 5B).

This lack of change in P2Y receptor function was correlated with the unaltered amount of $P2Y_{12}$ receptor RNA detected in Northern blots. When RNA had been extracted from non-differentiated PC12 cells, probes specific for $P2Y_1$, $P2Y_4$, and $P2Y_6$ gave no clear hybridization signal (not shown), although at least our probes for $P2Y_1$ and $P2Y_6$ are sensitive enough to detect the appropriate RNAs after isolation from primary cultures of rat sympathetic ganglia (Vartian *et al*., 2001). Thus, PC12 cells appear to express low levels of $P2Y_4$ and $P2Y_6$ only. The probes for $P2Y_2$ and $P2Y_{12}$ in contrast, gave robust hybridization signals. However, as revealed in Figure 5C, exposure of PC12 cells to NGF (50 ng ml$^{-1}$ for 5 days), did not alter the amount of $P2Y_{12}$ RNA, and the $P2Y_2$ receptor RNA, if anything, appeared weakly reduced after the NGF treatment.

## Discussion

Inhibition of adenylyl cyclase activity by metabotropic nucleotide receptors has been reported for a variety of tissues including, in particular, blood platelets, glioma cells, and endothelial cells. We now extend these findings to a neuronal cellular environment by showing that activation of P2Y receptors inhibits adenylyl cyclase activity in PC12 cells which are used as an experimental model to study the functions of neuronal receptors (Greene & Tischler, 1976). Previously, PC12 cells have been reported to express P2X and/or P2Y nucleotide receptors that mediate, for instance, ATP-gated cation currents (e.g. Nakazawa *et al*., 1990), stimulation of inositolphosphate synthesis (e.g. Murrin & Boarder, 1992), increases in intracellular $Ca^{2+}$ (e.g. Arslan *et al*., 2000), activation of mitogen activated protein kinase (Soltoff *et al*., 1998), inhibition of voltage-gated $Ca^{2+}$ channels (Vartian & Boehm, 2001), stimulation of neuritogenesis (D'Ambrosi *et al*., 2000), and induction of catecholamine secretion (e.g. Inoue *et al*., 1991).

In the present study, ADP and ATP did not alter basal cyclic AMP accumulation, but markedly modulated the production of this second messenger when triggered either by direct activation of the synthesizing enzyme adenylyl cyclase by forskolin or by activation of either endogenous adenosine $A_{2A}$ receptors or of heterologously expressed $\beta$-adrenoceptors. Surprisingly, micromolar concentrations of ADP affected stimulation-dependent cyclic AMP synthesis in opposite directions when forskolin and receptor agonists were used as stimulators, respectively: receptor-dependent cyclic AMP accumulation was reduced, whereas the forskolin-induced accumulation was enhanced by 10 – 100 $\mu$M ADP. In PC12 cell cultures, adenine nucleotides are rapidly degraded towards adenosine (Vollmayer *et al*., 2001). Since PC12 cells are known to express several subtypes of adenosine receptors with a functional predominance of the adenylyl cyclase-activating $A_{2A}$ receptor (Florio *et al*., 1999b), we assumed that adenosine was involved in the stimulatory effect of ADP. However, in the presence of adenosine deaminase, which was routinely included in the experimental solutions, exogenous adenosine at concentrations of up to 100 $\mu$M failed to stimulate adenylyl cyclase activity. Thus, the enhancing action was mediated by an adenine nucleotide rather than by the nucleoside.

The accumulation of cyclic AMP in PC12 cells stimulated by forskolin has been shown to involve activation of adenosine $A_{2A}$ receptors (Florio *et al*., 1999a). In accordance with this idea, the forskolin-induced cyclic AMP synthesis was reduced by the $A_{2A}$ receptor antagonist ZM 241385 in a concentration-dependent manner. Furthermore, the concentration-response curve for the stimulation of cyclic AMP accumulation by the $A_{2A}$ agonist CGS 21680 was shifted to the left by a factor of at least 10 in the presence of forskolin. Thus, in the presence of forskolin, the capability of $A_{2A}$ receptors to activate adenylyl cyclase is potentiated. This most likely reflects an enhanced affinity of adenylyl cyclase for activated G proteins $\alpha_s$ subunits in the presence of forskolin (Sunahara *et al*., 1995; Tesmer & Sprang, 1998; Simonds, 1999). As a consequence, agonists with low potency and/or efficacy at $A_{2A}$ receptors may stimulate adenylyl cyclase activity in the presence of forskolin. Recently, AMP has been shown to potentiate the forskolin-induced cyclic AMP accumulation in PC12 cells via $A_{2A}$ receptors (Florio *et al*., 1999b). Before, ATP and AMP had been reported to stimulate adenylyl cyclase activity *via* adenosine receptors in rat brain microvessels (Schütz *et al*., 1984), and at

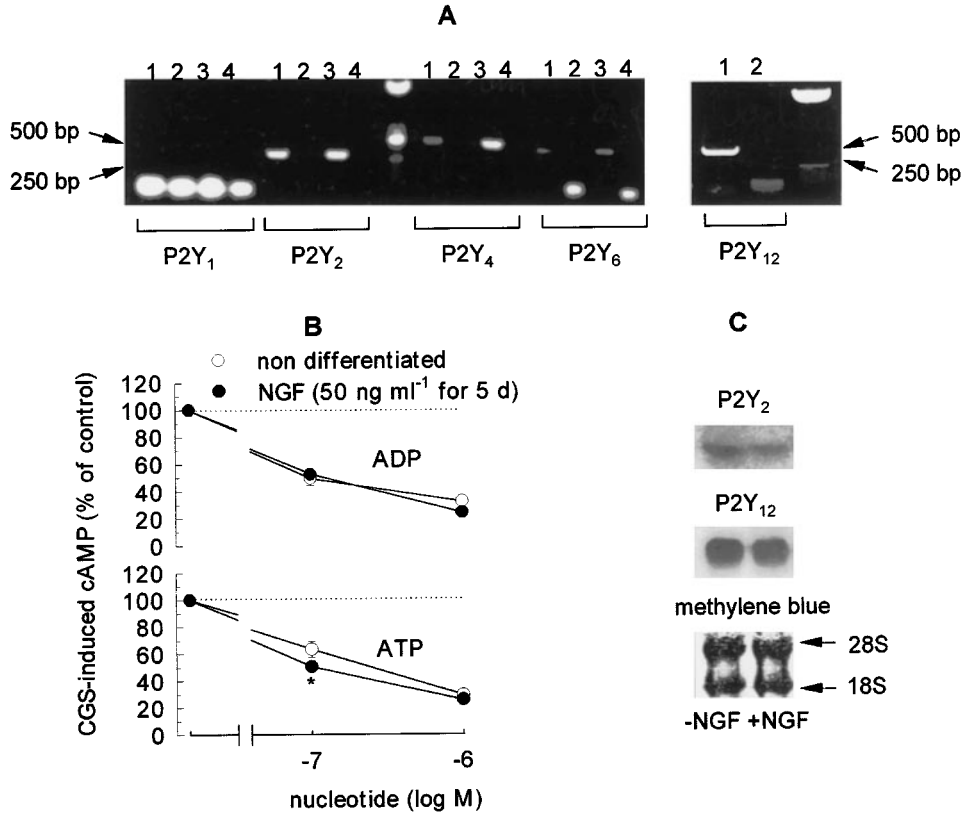


**Figure 5** Pattern of P2Y receptor expression in NGF-differentiated and non differentiated PC12 cells. (A) RT–PCR analysis of RNA extracted from two different clones (lanes 1 and 2 – clone EC; lanes 3 and 4 – clone OK) of PC12 cells (Note that all functional experiments have been performed on clone EC only). Primers and cycling conditions for the amplification of fragments of $P2Y_1$, $P2Y_2$ and $P2Y_4$ receptors were described by Harper *et al.* (1996) and for those of $P2Y_6$ by Arslan *et al.* (2000). The expected lengths of the appropriate PCR products are 558 bp ($P2Y_1$), 348 bp ($P2Y_2$), 382 bp ($P2Y_4$), and 347 bp ($P2Y_6$), respectively. A pair of primers (sense – ctttggcaacgaaaccaagt; antisense – atggtctggtggtcttcagg) was designed to amplify a 524 bp fragment of the rat $P2Y_{12}$ receptor. Amplification products were separated by electrophoresis through 2% agarose gels and stained with ethidium bromide. In lanes 2 and 4, the polymerase has been omitted during the reverse transcription step. (B) After loading with [$^3$H]-adenine, PC12 cells were incubated in RO 20-1724 (100 $\mu$M) for 105 min. During the last 15 min of this incubation period, 1 $\mu$M CGS 21680 and the indicated concentrations of ADP or ATP were also present. The amount of radioactivity retrieved within the fraction of cyclic AMP (cAMP) was calculated as percentage of the total radioactivity in the cell cultures, and values obtained in the presence of CGS 21680 were normalized to the data obtained in its absence (normalized to basal). Results obtained in the presence of ADP or ATP are expressed as percentage of those obtained in their absence (per cent of control; see Methods). Where indicated, PC12 cells have been exposed to NGF (50 ng ml$^{-1}$) for 5 days prior to the experiments. *Indicates significant differences between differentiated and non differentiated cultures at $P<0.05$ ($n=9$ in both cases). (C) Northern blot analysis performed with total RNA isolated from PC12 cell cultures (20 $\mu$g per lane) which had been incubated in the absence (-NGF) or presence (-NGF) of 50 ng ml$^{-1}$ NGF for 5 days prior to RNA extraction. The blot was consecutively probed with [$\alpha^{32}$P]-deoxycytidine triphosphate-labelled cDNA probes specific for $P2Y_2$ and $P2Y_{12}$. The bottom panel shows total RNA stained with methylene blue.

submillimolar concentrations, adenine nucleotides also interfere with the binding of CGS 21680 to striatal membranes (Pirotton & Boeynaems, 1993). In the present experiments, the enhancing action of ADP on forskolin-stimulated cyclic AMP synthesis was turned into an inhibition in the presence of the $A_{2A}$ receptor antagonist ZM 241385. Thus, the stimulatory effect of adenine nucleotides on adenylyl cyclase did not involve a nucleotide (P2), but rather an $A_{2A}$ nucleoside (P1), receptor.

PC12 cells express P2X as well as P2Y receptors (e.g. Arslan *et al.*, 2000). Some of the known P2Y receptors can be activated by uridine nucleotides, whereas P2X receptors cannot (Ralevic & Burnstock, 1998). The inhibitory effects of ADP and ATP on cyclic AMP accumulation were not mimicked by UDP or UTP. Therefore, the receptor involved might either be an uridine nucleotide-insensitive P2Y receptor or a P2X receptor. Activation of ATP-gated cation channels, i.e. of P2X receptors, might inhibit the activity of $Ca^{2+}$-sensitive adenylyl cyclase isoforms (Simonds, 1999) by mediating transmembrane $Ca^{2+}$ entry. However, removal of extracellular $Ca^{2+}$ did not prevent the reduction of cyclic AMP by ATP. $Ca^{2+}$-independent signalling mechanisms of P2X receptors were also not involved in the action of ATP, as P2X receptor blockade by suramin or PPADS (see Vartian & Boehm, 2001) failed to alter the inhibitory effect of the nucleotide. In contrast, the inhibition of adenylyl cyclase by ATP was rather augmented in the presence of these P2 receptor antagonists. The mechanisms underlying these enhancing actions are obscure, but one obvious factor are the reported inhibitory effects of suramin and PPADS on ectonucleotidase activity (Zimmerman, 2000). This type of action will support the effects of nucleotides mediated by receptors that are insensitive to these antagonists. Taken together, there was no evidence that the effects of ATP were

mediated by P2X receptors. In contrast, the involvement of G proteins, and thus of P2Y receptors, was directly demonstrated by the fact that pertussis toxin abolished the inhibition of adenylyl cyclase activity by ADP.

The cloned P2Y receptors of the rat that are sensitive to adenine, but insensitive to uridine, nucleotides comprise $P2Y_1$ and $P2Y_{12}$ (Ralevic & Burnstock, 1998; Hollopeter *et al*., 2001). $P2Y_1$ receptors are blocked by suramin and PPADS (Leon *et al*., 1997; Schachter, 1997), whereas the $P2Y_{12}$ receptors of C6 glioma cells (Jin *et al*., 2001) are PPADS-insensitive (Schachter *et al*., 1997) and only weakly antagonized by suramin (Boyer *et al*., 1994). Reactive blue 2, however, is quite a potent antagonist at the adenylyl cyclase inhibiting P2Y receptor of C6 glioma cells (Boyer *et al*., 1994). In the present study, only reactive blue 2, but not suramin or PPADS, blocked the inhibitory effect of ADP. Furthermore, the $P2Y_1$ preferring antagonist A3P5P (Boyer *et al*., 1996) failed to alter the action of ADP, but the $P2Y_{12}$ antagonist 2-methylthio-AMP (Hollopeter *et al*., 2001) displayed clearcut antagonistic activity. Likewise, ATPαS which blocks the adenylyl cyclase inhibiting nucleotide receptor in platelets (Hechler *et al*., 1998a), but activates $P2Y_1$ receptors (Vöhringer *et al*., 2000), attenuated the inhibitory action of ADP. Taken together, these pharmacological data indicate that adenine nucleotides inhibit adenylyl cyclase activity in PC12 cells *via* $P2Y_{12}$ receptors. This conclusion was also confirmed by our RT–PCR and Northern blot analyses: in line with previous results (Arslan *et al*., 2000), we did not detect any $P2Y_1$-specific transcripts, but considerable amounts of $P2Y_{12}$ mRNA. Amongst the other P2Y receptor subtypes that were found by RT–PCR (i.e. $P2Y_2$, $P2Y_4$, $P2Y_6$), only $P2Y_2$ was detectable in Northern blots and was thus expressed at high levels. Differentiation of PC12 cells with NGF did not alter the ADP- and ATP-dependent inhibition of adenylyl cyclase, nor did it alter the levels of $P2Y_{12}$ RNA as revealed by Northern blots. This again suggests that it was the $P2Y_{12}$ receptor subtype which mediated the inhibition of adenylyl cyclase activity.

Two previous reports indicated that nucleotides may affect adenylyl cyclase activity in PC12 cells (Yakushi *et al*., 1996; Murayama *et al*., 1998). These authors found that adenine nucleotides stimulated cyclic AMP accumulation at concentrations of 1–100 $\mu$M and inhibited cyclic AMP accumulation at $\geqslant$100 $\mu$M. However, most of these results had been obtained in the absence of adenosine deaminase and/or $A_{2A}$ receptor ligands. Thus, the enhancing effects of the nucleotides may have involved P1 ($A_{2A}$) receptors, as indicated by the present results. Furthermore, the inhibitory effects of submillimolar nucleotide concentrations could not be correlated with any of the known P2 receptor subtypes (Murayama *et al*., 1998): the reported rank order of agonist potencies was ATP$\gamma$S = 2-methylthio-ATP = ATP = ADP$\beta$S > benzoyl-ATP > > UTP > $\alpha,\beta$-methylene-ATP = $\beta,\gamma$-methylene ATP, and both, suramin and reactive blue 2 (each at 100 $\mu$M) failed to exert antagonistic activity. Nevertheless, these results indicate that PC12 cells may express additional P2Y receptor subtypes that contribute to the regulation of adenylyl cyclase activity by extracellular nucleotides. Most recently, a novel human P2Y receptor that inhibited adenylyl cyclase has been cloned and named $P2Y_{13}$ (Communi *et al*., 2001). This receptor was activated by nucleotides showing the following rank order of agonist potencies: ADP = 2-methylthio-ADP $\geqslant$ ADP$\beta$S > > ATP > UDP. Thus, by pharmacological criteria that receptor is different from $P2Y_{12}$ (Hollopeter *et al*., 2001) and from the receptor described here, since at these receptors 2-methylthio-ADP is 1000 fold more potent an agonist than ADP. Nevertheless, it remains to be established whether PC12 cells express the rat homologue of $P2Y_{13}$, which might then contribute to the nucleotide-dependent inhibition of adenylyl cyclase activity as reported above.

In conclusion, our results show that PC12 cells express $P2Y_{12}$ receptors which mediate an adenine nucleotide-dependent inhibition of cyclic AMP accumulation. Thus, the $P2Y_{12}$ receptor is not only present in blood platelets and glia cells (Hollopeter *et al*., 2001; Jin *et al*., 2001) but also in neuronal cells. There, it might also subserve cellular functions other than the mere control of cyclic AMP synthesis, for instance, the recently observed regulation of voltage-gated $Ca^{2+}$ channels and thus of presynaptic transmitter release (Vartian & Boehm, 2001).

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