P2Y-Receptors Mediating an Inhibition of the Evoked Entry of Calcium through N-Type Calcium Channels at Neuronal Processes

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Received April 23, 2002; accepted July 15, 2002

ABSTRACT
In the search for P2-receptors modulating the stimulation-evoked entry of calcium at processes of PC12 cells differentiated in the presence of nerve growth factor and neurotrophin-3, electrically evoked increases in free calcium were assessed by fura-2 microfluorimetry. Omission of calcium and addition of cadmium (100 μM) abolished or markedly reduced the evoked responses. The P2Y-receptor agonists 2-methylthio adenosine 5′-diphosphate (2-methylthio-ADP), ADP, and adenosine 5′-[O-(2-thiodiphosphate)] (300 nM), in contrast, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (10 μM), the selective P2Y1-receptor antagonist MRS 2179 (N^6-methyl-2′-deoxyadenosine 3′,5′-bisphosphate; 10 μM), as well as the adenosine A1-receptor antagonist DPCPX (8-cyclopentyl-1,3-dipropylxanthine; 100 nM), caused no change. Pre-treatment with pertussis toxin abolished the effect of ADP/βS. Reverse transcriptase-polymerase chain reaction revealed the presence of mRNA for P2Y12-receptors in nondifferentiated and differentiated PC12 cells. The results indicate that processes of differentiated PC12 cells possess P2Y12-receptors coupling to pertussis toxin-sensitive G-proteins and mediating an inhibition of the stimulation-evoked entry of calcium through ω-conotoxin GVIA-sensitive calcium channels. This suggests a role of P2Y12-receptors in neuromodulation in addition to their involvement in platelet aggregation.

The operation of P2-receptors inhibiting the release of neurotransmitters has been demonstrated in the peripheral and central nervous system (for reviews see Silinsky et al., 1998; Inoue et al., 1999; von Kügelen et al., 1999a). At postsynaptic symmetric axons, the receptors are activated by endogenous adenine nucleotides released as cotransmitters of norepinephrine and, hence, mediate a negative feedback inhibition of synaptic transmitter release (Fuder and Muth, 1993; von Kügelen et al., 1993). Release-inhibiting P2-receptors have also been found in adrenal chromaffin cells and the cell bodies of rat PC12 cells (Gandia et al., 1993; Currie and Fox, 1996; Powell et al., 2000; Vartian and Boehm, 2001; Unterberger et al., 2002). The release-inhibiting P2-receptors share some properties with cloned and expressed P2Y1-receptors (for references see above), but their molecular identity is yet not known. In addition to inhibitory P2-receptors, the cell bodies of postsynaptic sympathetic neurons, adrenal chromaffin cells, and PC12 cells possess excitatory P2-receptors of distinct subtypes and with distinct signaling transduction pathways (for reviews see Silinsky et al., 1998; von Kügelen et al., 1999a; for PC12 cells see, for example, Arslan et al., 2000; Unterberger et al., 2002).

The inhibition of calcium channels at axon terminals is a key element in the modulation of transmitter release by inhibitory G-protein-coupled receptors (see Przywara et al., 1993; Mirotznik et al., 2000; Jarvis and Zamponi, 2001). Therefore, we searched for P2-receptors modulating the elec-
tricly evoked entry of calcium in processes of differentiated PC12 cells. Effects of P2-receptor agonists and antagonists on the electrically evoked increases in free calcium concentration in these processes were assessed by means of fura-2 microfluorimetry to characterize P2-receptor subtypes in this model system. A contribution of different calcium channels to the evoked calcium entry and the possible involvement of pertussis toxin-sensitive G-proteins were also analyzed. Moreover, we searched for the presence of mRNA encoding for the recently cloned P2Y12-receptor in PC12 cells. A part of the results has been presented at a meeting of the Federation of the European Pharmacological Societies (Kulick and von Kügelgen, 2001).

**Materials and Methods**

**Culturing.** PC12 cells (European Collection of Cell Cultures, Salisbury, UK; original passage CB 2745) were cultured at 37°C and 5% CO₂ in RPMI 1640 medium (11835; Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal bovine serum (heat-inactivated; 10108; Invitrogen), 5% horse serum (heat-inactivated; 26050; Invitrogen), 12.5 U/ml penicillin, and 12.5 µg/ml streptomycin (15140; Invitrogen). The cells were split once a week by treating with tryps-EDTA (0.5 g/l; 25300; Invitrogen) and triturating with a fire-polished Pasteur pipette. Cells from passages 3 to 30 were used for further experiments. About 20,000 dissociated cells in 50 µl of medium were poured onto coverslips (25 mm in diameter; 12-545-86; Fisher Scientific, Schwerte, Germany) coated first with poly(L-lysine) (0.01%; P4707; Sigma Chemie, Schnelldorf, Germany) and then with Matrigel (356234; BD Biosciences, Bedford, MA). The coverslips were placed in a 35-mm culture dish (Greiner, Solingen, Germany) and incubated for 30 to 45 min. Then culture medium, 1.5 ml, was added. The medium (Dulbecko’s modified Eagle’s medium/nutri-ent mixture F12, 1:1; 11039, Invitrogen) was supplemented with N2 supplement (1×; 17502; Invitrogen), Glutamax I (1×; 35050; Invitro- gen), 25 U/ml penicillin, 25 µg/ml streptomycin, and, to induce neuronal differentiation, 40 ng/ml nerve growth factor-β (human, recom- binant; N1408; Sigma), as well as 10 ng/ml neurotrophin-3 (human, recombinant; N1905; Sigma). The cells were cultured for 6 to 18 days at 37°C, 5% CO₂ medium was exchanged every 2 to 3 days.

**fura-2 Microfluorimetry.** Cells cultured on coverslips were incu-bated with fura-2 acetoxymethyl ester (fura-2 AM, 2 µM; F-1225; Molecular Probes Europe, Leiden, Netherlands) for 30 min at 37°C. A coverslip was then fixed in a superfusion chamber between two platinum electrodes and mounted on the stage of a Zeiss Axiolvert microscope equipped with an oil immersion P-Fluar 40× objective (Zeiss, Jena, Germany). The cells were superfused at 1 ml/min and room temperature with buffer containing 135 mM NaCl, 4.8 mM KCl, 1.3 mM CaCl₂, 1.2 mM KH₂PO₄, 10 mM glucose, and 10 mM HEPES (pH adjusted to 7.4 using NaOH). KH₂PO₄ was omitted in experiments with cadmium. After a presuperfusion period of 30 min, fluorescence emission was measured after alternating excitation at 340 nm and 380 nm each applied for 5 to 9 ms and repeated every 1 s using a Polychrome II monochromator, an Imago charge-coupled device camera, and the TILLvisION imaging system (Till Photonics, Planeg, Germany). Measurements were performed over processes (see Fig. 1A). One area in one process was evaluated per cell. The portion of the coverslip depicted by the charge-coupled device camera contained processes of one to three individual cells. Preparations were stimulated by trains of 10 electrical pulses (p) at 100 Hz (200 mA, 0.3 ms; Stimulator II; Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany) applied every 3 min. After three preceding periods of stimulation in the absence of drugs, drugs were added to the buffer for 6 min. When used, antagonists were given 18 min before the agonist. In the intervals between stimulation periods, the cells were protected from the excitation light to prevent bleaching and phototoxicity (see gaps in the traces in Figs. 1C and 2).

The ratio fluorescence emission due to excitation at 340 nm/fluorescence emission due to excitation at 380 nm (F 340 nm/F 380 nm) was evaluated for estimation of changes in intracellular calcium concentration. Due to the known problems of calibration (see Leipziger et al., 1991), the values were not transformed in absolute calcium concentrations. Stimulation-evoked increases in the ratio were expressed as the difference fluorescence ratio at peak minus basal ratio (determined by averaging the four ratios obtained before onset of the respective stimulation period). Experiments with a high vari-ability in the responses to the preceding periods of electrical stimu-lation (difference of a response >20% from the mean of the three respective responses) were excluded from further evaluation. For the quantification of the experimentally induced changes, the first re-sponse to stimulation in the presence of a drug (2 min after addition) was expressed as percentage of the averaged responses to the three preceding stimulation periods (% of preceding responses).

**Isolation of poly(A⁺) mRNA and RT-PCR.** mRNA was isolated from nondifferentiated and differentiated cells cultured as described above, using the Oligotex Direct mRNA Mini Kit (72022; QIAGEN GmbH, Hilden, Germany). The efficiency of the isolation was proved by the demonstration of mRNA for β-actin. Reverse transcriptase-poly-merase chain reaction (RT-PCR) was performed using gene-specific primers for the rat P2Y12 receptor (sense primer: CACATGGAGGTGCCGTGCTGCCAAC; antisense primer: CATGGGGTTCCTGCTCTTGTC) and β-actin (sense: GTACCCCATTTGAACAGCATG; anti-sense: GTTCAGGACACATTGCTTCCAG) and the Superscript One Step RT-PCR kit (Invitrogen). Annealing temperature for the PCR reaction was 67.7°C (30 cycles) in the case of the undifferentiated PC12 cells and 66°C (45 cycles) in the case of the differentiated PC12 cells.
when amplifying the mRNA for the P2Y12 receptor and β-actin. For control experiments the enzyme reverse transcriptase was omitted (instead of the RT-PCR enzyme mix, Platinum Polymerase was used; Invitrogen). PCR products were analyzed by ethidium bromide staining after agarose (0.9%) gel electrophoresis. The product of one RT-PCR reaction with primers for the P2Y12 receptor was cloned into the vector pGEM3Z/F-Topo (Promega, Madison, WI) using a LICOR Gene-Vציות labeled primer cycle sequencing kit; Amersham Biosciences UK, Little Chalfont, Buckinghamshire, UK) by an analysis of variance (ANOVA) followed by the Bonferroni’s multiple comparison test (Prism, GraphPad, San Diego CA). P < 0.05 or lower was the significance criterion.

Results

PC12 cells were plated on coverslips coated with poly(l-lysine) and Matrigel and cultured in the presence of nerve growth factor-β (40 ng/ml) and neurotrophin-3 (10 ng/ml) for up to 18 days. After about 6 days in culture, the cells developed long and multiple processes as shown in Fig. 1A and B. Fura-2 fluorescence was measured as an estimate of changes in intracellular calcium concentration. After incubation with fura-2 AM, fluorescence was observed not only in cell bodies (not shown) but also in the processes (Fig. 1B). One area of one process was evaluated per cell (see marked area in Fig. 1A). Electrical stimulation by square pulses (0.3 ms) caused transient increases in the fura-2 fluorescence ratio (Fig. 1C) dependent on the number of pulses applied (not shown). When the preparations were stimulated by periods of 10 pulses/100 Hz applied every 3 min, the responses were well reproducible (Fig. 1C). In the absence of drugs, the response to a fourth period of stimulation amounted to 106.9% of the averaged responses to the three preceding periods (control; see legend to Table 1). On average, a preceding period of stimulation caused an increase in the fura-2 fluorescence ratio by 64.0 ± 4.1% above baseline (n = 180).

Effects of Calcium Channel Blockers. Omission of calcium from the superfusion buffer abolished the response to electrical stimulation (reduction by 92.6 ± 2.4%; n = 5; P < 0.01). To study the contribution of voltage-dependent calcium channels to the evoked increase in calcium concentration, effects of several channel blockers were tested. Cadmium (100 μM) as well as the N-type calcium channel blocker ω-conotoxin GVIA (0.5 μM; Hirning et al., 1988) markedly reduced the responses to stimulation (Fig. 1C and Table 1), whereas the L-type calcium channel blocker nifedipine (10 μM) and the P/Q-type calcium channel blocker ω-agatoxin

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<th>TABLE 1</th>
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| Effects of calcium channel blockers on the electrically evoked responses

<table>
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<tr>
<th>Compound</th>
<th>% of respective control</th>
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<tbody>
<tr>
<td>Control</td>
<td>100.0 ± 3.1 (24)</td>
</tr>
<tr>
<td>Cd²⁺ (100 μM)</td>
<td>18.9 ± 4.5 (6)**</td>
</tr>
<tr>
<td>Agatoxin IVa (0.5 μM)</td>
<td>96.2 ± 2.6 (9)</td>
</tr>
<tr>
<td>Conotoxin GVIA (0.5 μM)</td>
<td>32.3 ± 12.8 (6)**</td>
</tr>
<tr>
<td>Control plus DMSO</td>
<td>100.0 ± 5.3 (6)</td>
</tr>
<tr>
<td>Nifedipine (10 μM)</td>
<td>104.1 ± 14.8 (5)</td>
</tr>
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</table>

** P < 0.01 versus respective control.
IVa (0.5 μM; Mintz et al., 1992) failed to alter the responses (Table 1). None of the compounds changed basal fluorescence ratios (not shown).

**Effects of Adenosine and Nucleotides.** Next, the effects of P1- and P2-receptor agonists were analyzed. The P2Y-receptor agonist ADPβS (Ralevic and Burnstock, 1998) has previously been shown to cause a preferential activation of presynaptic P2-receptors (see von Kügelgen et al., 1999a). When used in the present study on processes of differentiated PC12 cells at a concentration of 30 μM, ADPβS attenuated the electrically evoked responses by 51%, 2 min after addition of the compound (Figs. 2A and 3). On average, the inhibitory effect of ADPβS (30 μM) was smaller when the compound was present for an additional period of 3 min (i.e., for two periods of stimulation; inhibition by 34.4 ± 5.6%, n = 11; P < 0.01, paired t test; e.g., Fig. 2A). For this reason, effects observed 2 min after addition of drugs were analyzed in the present study. The inhibition by ADPβS was reversible after removal of the compound from the superfusion buffer (Fig. 2A) and dependent on the concentration used (Fig. 3). ADP and 2-methylthio-ADP, agonists at the P2Y1-, P2Y12-, and P2Y13-receptors (von Kügelgen and Wetter 2000; Comminu et al., 2001; Hollopeter et al., 2001; Zhang et al., 2001), also caused an inhibition of the electrically induced increases in fura-2 fluorescence (Fig. 3). 2-Methylthio-ADP was the most potent compound followed by ADP and ADPβS. In contrast to the adenine nucleotides tested, both the diadenine nucleotide Ap4A, an agonist at the rat P2Y2- and P2Y4-receptors (von Kügelgen and Wetter, 2000), and the P1-purinoceptor agonist adenosine failed to change the electrically evoked responses (each compound tested at 30 μM; Fig. 3). The same was true for the uracil nucleotides UDP (30 μM; Fig. 3) and UTP (evoked response in the presence of 30 μM: 95.0 ± 2.4% of control, n = 7). UDP and UTP are known to activate the P2Y2-, P2Y4-, and P2Y6-receptors (Nicholas et al., 1996). None of the compounds described above affected the basal fluorescence ratio. However, ATP (30 μM), which activates P2X-receptors in addition to P2Y-receptors (North and Barnard, 1997; Ralevic and Burnstock, 1998; Nörenberg and Illes, 2000), increased the basal ratio by 49.5 ± 21.6% (n = 5, P < 0.01). This increase is likely to be due to a P2X-receptor-mediated influx of calcium (see Arslan et al., 2000; Nörenberg and Illes, 2000). ATP also tended to reduce the electrically evoked responses in the present study on processes of PC12 cells, but the effect was not further analyzed due to the marked increase in basal ratio, which makes the determination of electrically evoked responses unreliable.

**Interaction with Purinoceptor Antagonists.** Antagonists were given 18 min before the agonist ADPβS (30 μM). DPCPX used at a concentration (0.1 μM) about 200 times its affinity constant at the adenosine A1-receptor (Lohse et al., 1987) did not affect the inhibitory action of ADPβS (Fig. 4). In contrast, reactive blue 2 (RB2, 3 μM) and suramin (100 μM), both of which block a number of P2-receptor subtypes, abolished the inhibitory effect of ADPβS. The P2-antagonist PPADS, known to effectively block P2Y1-receptors in addition to several P2X-subtypes (Lambrecht, 2000; von Kügelgen and Wetter, 2000), had no significant effect when used at a concentration of 10 μM.

In addition to reactive blue 2, suramin, and PPADS, four P2-antagonists with a higher selectivity for distinct receptor subtypes were tested. The preferential P2Y1-receptor antagonist MRS 2179, used at 10 μM (about 100 times its affinity constant at the P2Y1-receptor; Boyer et al., 1998), failed to alter the effect of ADPβS. In contrast, CMPS (1 μM), 2-methylthio-AMP (10 μM), and AR-C 69931MX (300 nM), all of which have been shown to block the recently cloned P2Y12-receptor (Hollopeter et al., 2001; Takasaki et al., 2001), abolished the action of ADPβS (Fig. 4). None of the antagonists themselves changed the basal fluorescence ratio or the electrically evoked responses (not shown).

![Fig. 3. Effects of ADP, ADPβS, 2-methylthio-ADP (2MeSADP), Ap4A, UDP, and adenosine (Ade) on the electrically induced responses. The figure shows the statistical evaluation of the responses 2 min after addition of the compounds. Responses were expressed as percentage of the averaged responses to the three preceding periods of stimulation (% of preceding responses). Means ± S.E. of n = 4 to 24. *, **, P < 0.05 and P < 0.01, respectively, versus control.](image)

![Fig. 4. Interaction with the adenosine A1-receptor antagonist DPCPX and several P2-receptor antagonists [RB2, suramin (Sur), PPADS, MRS 2179 (MRS), CMPS, 2-methylthio-AMP (MeS), and AR-C 69931MX (AR-C)]. Antagonists were added 18 min before the agonist. The figure shows the statistical evaluation of the responses 2 min after addition of ADPβS (30 μM). Responses were expressed as % of respective control (for details, see legend to Table 1). Means ± S.E. of n = 4 to 24. *, **, #, ##, P < 0.05 and P < 0.01 versus respective control; #, ##, P < 0.05 and P < 0.01, respectively, versus responses in the absence of antagonists.](image)
Effect of Treatment with Pertussis Toxin. To analyze a possible coupling of the inhibitory receptor to pertussis toxin-sensitive G-proteins, cells were treated with pertussis toxin (200 ng/ml) or its solvent for 16 to 22 h. Treatment with pertussis toxin abolished the effect of ADPβS (Fig. 2B; Table 2) without any change in the basal fluorescence ratio or the responses to the preceding periods of stimulation (not shown).

Presence of mRNA for P2Y12-Receptors in PC12 Cells. RT-PCR with primers specific for the coding sequence of the rat P2Y12-receptor revealed products of the expected length from two mRNA preparations of nondifferentiated PC12 cells (not shown). Control reactions without the enzyme reverse transcriptase showed no products, confirming that mRNA but not genomic DNA acted as a template for the PCR reaction. Cycle sequencing of one RT-PCR reaction product confirmed the identity of the sequence with that of the cloned rat P2Y12-receptor (Hollopeter et al., 2001; GenBank accession code AF313450). When using three mRNA preparations from differentiated PC12 cells cultured on coverslips in the presence of nerve growth factor-β and neurtrophin-3 (see Materials and Methods), similar results were obtained (Fig. 5).

Discussion

In the present study we searched for P2-receptors inhibiting the influx of calcium via voltage-dependent calcium channels in processes of differentiated PC12 cells. Changes in the concentration of calcium in the processes were assessed by fura-2 microfluorimetry. Due to the known problems of calibration (Leipziger et al., 1991), fura-2 fluorescence ratios (F 340 nm/F 380 nm) instead of absolute concentrations of calcium were statistically evaluated.

Electrically Evoked Calcium Entry through N-Type Calcium Channels. Electrical field stimulation of the preparations induced an influx of calcium in the processes through voltage-dependent calcium channels as shown by the blockade of the responses by either omission of calcium from the buffer or addition of cadmium to the buffer. The evoked increase in calcium concentration was also markedly affected by ω-conotoxin GVIA, but not nifedipine or ω-agatoxin IVa, indicating that the increase was at least predominantly mediated by an influx of calcium ions through N-type (Ca2.2; Ertel et al., 2000) calcium channels (for references, see above). N-type calcium channels also play the predominant role in stimulation-evoked calcium entry in postganglionic sympathetic neurons (e.g., Hirning et al., 1988; von Kügelgen et al., 1999b). In contrast, in adrenal chromaffin cells and cell bodies of PC12 cells, L-type and P/Q-type channels are involved in addition to the N-type channels in the evoked entry of calcium (Liu et al., 1996; Lukyanetz and Neher, 1999).

Inhibitory P2Y12-Like Receptors Coupling to Pertussis Toxin-Sensitive G-Proteins. The present study demonstrates the operation of P2Y12-like receptors inhibiting the electrically evoked entry of calcium in processes of differentiated PC12 cells. The preferential P2Y-receptor agonists ADPβS, ADP, and 2-methylthio-ADP inhibited the evoked entry of calcium in a concentration-dependent manner. The receptor involved in the inhibition of calcium entry at the processes of PC12 cells is not an adenosine receptor, but a P2-receptor, as shown by: 1) the lack of any effect of adenosine, 2) the failure of the adenosine A1-receptor antagonist DPCPX to alter the effect of ADPβS, and 3) the blockade of the effect of ADPβS by several P2-receptor antagonists including the selective P2Y-antagonist AR-C 69931MX. These findings also exclude an involvement of the recently described heteromers of adenosine A1-receptors and P2Y1-receptors (Yoshio et al., 2001) or the putative P3-purinoceptors (Shiozuka et al., 1988) since both the heteromers, and P3-purinoceptors have been found to be sensitive to the blockade by xanthine derivatives such as DPCPX.

Which subtype of P2-receptor mediates the inhibitory effect? P2X-receptors are ligand-gated ion channels, whereas P2Y-receptors belong to the family of G-protein-coupled receptors (North and Barnard, 1997; Ralevic and Burnstock, 1998). The blockade of the effect of ADPβS by the treatment of the cells with pertussis toxin indicates that the receptors couple to Gαi-proteins and clearly identifies the receptors as P2Y-receptors. The lack of any effect of UDP, UTP, and Ap4A excludes the involvement of P2Y2-, P2Y4-, and P2Y6-receptors (for review see Ralevic and Burnstock, 1998; von Kügelgen and Wetter, 2000). The action of the diphosphate nucleotides ADPβS, ADP, and 2-methylthio-ADP is compatible with an involvement of a diphosphate nucleotide preferring P2Y-receptor, i.e., the P2Y1-, P2Y12-, or P2Y13-receptor (Ralevic and Burnstock, 1998; von Kügelgen and Wetter, 2000; Communi et al., 2001; Hollopeter et al., 2001), but argues against an involvement of the triphosphate nucleotide preferring P2Y11-receptor (Communi et al., 1997). The lack of any interaction of ADPβS with the preferential P2Y1-receptor antagonist MRS 2179 (Boyer et al., 1998) excludes a characterization as P2Y1-receptor. Moreover, the blockade of the effect of ADPβS by p-chloromercuriphenyl sulfonic acid

**TABLE 2**

<table>
<thead>
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<th>Treated with Solvent (n)</th>
<th>Treated with Pertussis Toxin, 200 ng/ml (n)</th>
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<tbody>
<tr>
<td><strong>% of respective control</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>100.0 ± 3.1 (6)</td>
<td>100.0 ± 4.8 (7)</td>
</tr>
<tr>
<td>ADPβS (30 μM)</td>
<td>48.4 ± 5.3 (6)***</td>
<td>96.8 ± 6.4 (4)***</td>
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</table>

***P < 0.01 versus respective control. **P < 0.01 versus responses observed in cells treated with solvent.
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and 2-methylthio-AMP, as well as AR-C 69931MX, used at a nanomolar concentration strongly suggests the characterization as P2Y12-receptor since these antagonists have previously been shown to block the cloned and expressed P2Y2-receptor (Hollopeter et al., 2001; Takasaki et al., 2001). This view is further confirmed by the observation that both the cloned and expressed P2Y12-receptor as well as the inhibitory P2Y-receptor at processes of PC12 cells studied in the present experiments were sensitive to the blockade by suramin and reactive blue 2 but not by PPADS (see Fig. 4 of the present article and Fig. 3A of Takasaki et al., 2001). The cloned and expressed P2Y12-receptor has been shown to couple to a pertussis toxin-sensitive G-protein (Hollopeter et al., 2001; Zhang et al., 2001). The same appears to be true for the inhibitory P2Y-receptor in PC12 cells in agreement with a characterization of this receptor as P2Y12. Due to the fact that the pharmacology of the recently cloned human P2Y13-receptor is yet not well defined (and that the rat ortholog of this receptor has yet not been cloned), an additional contribution of a P2Y13-receptor to the observed effects cannot be excluded.

The properties of the inhibitory P2Y12-like receptors found in the present study at the processes of differentiated PC12 cells seem to be very similar to or identical with those of release-inhibiting P2-receptors operating in the peripheral and central nervous system as well as at adrenal chromaffin cells or the cell bodies of PC12 cells (for references, see the introduction). When tested in these studies, ADP, ADPβS, 2-methylthio-ADP, and ATP acted as agonists, whereas uracil nucleotides were ineffective (for references, see the introduction). Other common features are the blockade by the antagonists suramin and reactive blue 2 as well as by pre-treatment with pertussis toxin. Moreover, in a very recent study published during the preparation of this article, the P2-receptors inhibiting adenylyl cyclase activity in PC12 cells were characterized as P2Y12-receptors (Unterberger et al., 2002). Similarly, as in our study, 2-methylthio-ADP was more potent than ADP in inhibiting adenylyl cyclase activity, and the agonist-induced inhibition was blocked by the antagonists 2-methylthio-AMP and reactive blue 2 but not by PPADS. In contrast to our results and to the results obtained by Takasaki et al. (2001) at the cloned and expressed P2Y12-receptor, suramin (100 μM) failed to act as an antagonist at the receptor inhibiting adenylyl cyclase activity (Unterberger et al., 2002). The reason for the difference is not known.

Except for ATP, which is likely to activate P2X-receptors, none of the nucleotides tested caused an increase in basal calcium concentration in the processes of the PC12 cells (see Results and Fig. 2). Hence, the processes appear to possess no excitatory P2Y-receptors, which have previously been shown to operate at the cell bodies of PC12 cells and which are sensitive to UTP in addition to ATP (see, for example, Arslan et al., 2000).

Presence of mRNA for P2Y12-Receptors in PC12 Cells. In agreement with the idea that PC12 cells express P2Y12-receptors, RT-PCR experiments using poly(A+) mRNA preparations from nondifferentiated and differentiated PC12 cells showed the presence of mRNA for the full coding sequence of the P2Y12-receptor, compatible with an expression of these receptors in PC12 cells.

A Role for P2Y12-Receptors as Inhibitory Receptors in the Nervous System? P2Y12-receptors have been cloned from platelets (Hollopeter et al., 2001) and have been identified as one of the P2-receptors involved in ADP-induced platelet aggregation (Hollopeter et al., 2001; Takasaki et al., 2001; Zhang et al., 2001). The present results suggest an additional functional role for P2Y12-receptors: a role as inhibitory receptors operating at neuronal cells. Since mRNA for P2Y2-receptors seems to be abundantly expressed in the brain (Hollopeter et al., 2001; Takasaki et al., 2001; Zhang et al., 2001), the role of P2Y12-receptors as inhibitory receptors in the nervous system may be widespread. A recent study using guanosine 5'-O-(3-thio)triphosphate autoradiography on rat brain sections also showed evidence for the operation of a receptor similar to the platelet ADP receptor in the gray and white matter of the forebrain and brainstem (Lahtinen et al., 2001).

The present results indicate that P2Y12-receptors mediate inhibitory effects of adenine nucleotides at processes of PC12 cells. Remaining questions are whether P2Y12-receptors or distinct P2Y-subtypes such as the P2Y1-receptor or the P2Y13-receptor are involved in neuromodulation at peripheral and central neurons and whether the receptors couple to calcium or potassium channels (cf. Fernández-Fernández et al., 2001).

Conclusions

The present study directly demonstrates the operation of inhibitory P2Y2-receptors at neuronal processes. The receptors mediate an inhibition of the stimulation-evoked entry of calcium through N-type calcium channels. The properties of the receptors suggest a characterization as P2Y12-receptors.

Acknowledgments

We thank Prof. Dr. P. Illes (Rudolf-Boehm-Institut f¨ur Pharmakologie und Toxikologie, Universit¨at Leipzig, Leipzig, Germany) and Prof. Dr. H. J. Ruoff (Bayer, Fachbereich Klinische Forschung, Wuppertal, Germany) for the supply of drugs.

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