

Autoregulation in PC12 cells via P2Y receptors: Evidence for non-exocytotic nucleotide release from neuroendocrine cells

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Abstract Nucleotides are released not only from neurons, but also from various other types of cells including fibroblasts, epithelial, endothelial and glial cells. While ATP release from non-neural cells is frequently Ca^{2+} independent and mostly non-vesicular, neuronal ATP release is generally believed to occur via exocytosis. To evaluate whether nucleotide release from neuroendocrine cells might involve a non-vesicular component, the auto-crine/paracrine activation of P2Y₁₂ receptors was used as a biosensor for nucleotide release from PC12 cells. Expression of a plasmid coding for the botulinum toxin C1 light chain led to a decrease in syntaxin 1 detected in immunoblots of PC12 membranes. In parallel, spontaneous as well as depolarization-evoked release of previously incorporated [³H]noradrenaline from transfected cells was significantly reduced in comparison with the release from untransfected cells, thus indicating that exocytosis was impaired. In PC12 cells expressing the botulinum toxin C1 light chain, ADP reduced cyclic AMP synthesis to the same extent as in non-transfected cells. Likewise, the enhancement of cyclic AMP synthesis either due to the blockade of P2Y₁₂ receptors or due to the degradation of extracellular nucleotides by apyrase was not different between non-transfected and botulinum toxin C1 light chain expressing

cells. However, the inhibition of cyclic AMP synthesis caused by depolarization-evoked release of endogenous nucleotides was either abolished or greatly reduced in cells expressing the botulinum toxin C1 light chain. Together, these results show that spontaneous nucleotide release from neuroendocrine cells may occur independently of vesicle exocytosis, whereas depolarization-evoked nucleotide release relies predominantly on exocytotic mechanisms.

Keywords Cyclic AMP · Nucleotide release · PC12 cells · P2Y receptor · Vesicle exocytosis

Introduction

Adenine and uridine nucleotides are present in and released from all types of cells [2] and exert effects via P2X and/or P2Y receptors: P2X receptors are ATP-gated cation channels [25]; P2Y receptors are G protein-coupled receptors, and eight different types have been identified (P2Y₁, 2, 4, 6, 11, 12, 13, 14; [1]). P2Y₁, 11, 12 and 13 are activated by adenine nucleotides, whereas P2Y₆ is activated by uridine nucleotides. P2Y₂ and 4 are sensitive to adenine and uridine nucleotides, and P2Y₁₄ is activated by UDP-glucose. In a large number of non-neural tissues, e.g. liver, kidney, bones and blood vessels, released nucleotides subserve autocrine/paracrine functions by activating certain P2Y receptors [32, 16, 38, 12, 30]. In a given cell, this autocrine/paracrine regulation depends on the release of nucleotides, the presence of P2Y receptors and occasionally on the enzymatic activities of nucleotidases: in hepatoma cells [16], for instance, released ATP is degraded to ADP to stimulate P2Y₁ receptors; in epithelial [32] and osteoblastic [38] cells, in contrast, ATP itself activates P2Y₁₁ and P2Y₂ receptors, respectively.

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Although ATP has first been described to be released from neurons [15] and to act as a neurotransmitter [6], available information about neuronal autoregulation via released nucleotides and P2Y receptors is limited. Early examples were the feedback inhibition of sympathetic transmitter release via presynaptic P2Y receptors (see [4]) and the ATP-mediated autocrine inhibition of voltage-activated Ca^{2+} channels in bovine chromaffin cells [9]. More recently, autoregulation via P2Y receptors has been described for the pheochromocytoma cell line PC12 [24]. There, endogenous P2Y₁₂ receptors were activated by spontaneous nucleotide release to achieve half-maximal inhibition of cyclic AMP (cAMP) synthesis. Depolarization of PC12 cells by 100 mM K^+ further enhanced nucleotide release and thereby led to full inhibition of cAMP generation. This latter effect was prevented when voltage-activated Ca^{2+} channels were blocked by Cd^{2+} or when extracellular nucleotides were degraded by apyrase. Thus, the inhibition of cAMP synthesis in PC12 cells via P2Y receptors can be viewed as a biosensor for nucleotide release [24].

Nucleotides are released from various cells including fibroblast-like, epithelial, endothelial, glial and neuronal cells [36, 28, 21, 26, 35]. ATP release from non-neural cells can be elicited by different stimuli, such as hypotonic solutions [36], mechanical stimulation [28] or exchange of culture media [21, 26] and is frequently Ca^{2+} independent and therefore most probably non-vesicular. The mechanisms underlying this non-vesicular ATP release remained largely elusive, but ATP binding cassette transporters, stretch-activated cation channels as well as connexin hemichannels have been implicated [22]. In addition, evidence has been presented that indicates that nucleotide release from non-neural cells may to some extent be vesicular (e.g. [17, 8]).

In neurons and neuroendocrine cells, ATP is stored in vesicles either alone or together with classic neurotransmitters and is thus released by exocytosis [27]. Vesicle exocytosis occurs spontaneously at a slow rate and is largely accelerated when Ca^{2+} entry is triggered by depolarization-induced opening of voltage-activated Ca^{2+} channels [29]. Accordingly, depolarization-evoked ATP release from neurons and neuroendocrine cells is entirely Ca^{2+} dependent [35, 13], but it remained unknown whether all of the spontaneous nucleotide release from neuronal sources is also vesicular.

Vesicle exocytosis in neurons and neuroendocrine cells involves the SNARE proteins syntaxin, SNAP-25 and VAMP/synaptobrevin. These proteins are cleaved by various serotypes of clostridial neurotoxins which thereby prevent exocytosis. In particular, botulinum neurotoxin C1 cleaves syntaxin, and in some cells also SNAP-25, and thereby impedes spontaneous as well as Ca^{2+} -evoked vesicle exocytosis [5]. Transfection of PC12 cells with a

plasmid coding for the botulinum toxin C1 light chain was reported to largely reduce spontaneous as well as depolarization-evoked release of co-transfected human growth hormone [10]. Here, we used an analogous method to investigate whether in PC12 cells the activation of P2Y₁₂ receptors by endogenously released nucleotides relies on vesicle exocytosis.

Materials and methods

Materials

[2,8-³H]adenine (specific activity 32 Ci mmol⁻¹) and levo-[ring-2,5,6-³H]noradrenaline (specific activity 37 Ci mmol⁻¹) were obtained from NEN (Vienna, Austria). Na-ADP, 4-(3-butoxy-4-methoxybenzyl)imidazoline-2-one (RO 20-1724), 3',5'-cyclic AMP, apyrase (grade VII, with an approximately 1:1 ratio in ATPase and ADPase activity), 2-methylthio-AMP, and 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamido-adenosine (CGS 21680) were purchased from Sigma (Vienna, Austria).

Cell culture and transfection

PC12 cells were maintained and subcultured as previously described in detail [24]. The cells were plated onto collagen-coated (Biomedical Technologies Inc., Stoughton, MA, USA) culture dishes (NUNC, Roskilde, Denmark) and were kept in OptiMEM (InVitrogen, 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% fetal calf serum and 10% horse serum (both InVitrogen, Vienna, Austria). Once per week, cell cultures were split; the medium was exchanged twice per week. To investigate the outflow of previously incorporated [³H]noradrenaline under continuous superfusion, PC12 cells were plated onto 5-mm discs, for assays of cAMP accumulation they were plated onto 6-well culture dishes and for membrane preparations for Western blots they were plated onto 100-mm culture dishes. All this tissue culture plastic was coated with collagen (as above).

For the generation of PC12 cell clones stably expressing the botulinum toxin C1 light chain (BoNT/C1), 15 µg of a pcDNA3 vector harbouring the botulinum toxin C1 light chain (pBoNT/C1; kindly provided by R.D. Burgoyne, Liverpool, UK) [10] were mixed with 50 µl of the TransFast transfection reagent (Promega, Mannheim, Germany) and added to semiconfluent PC12 cell cultures in serum-free medium. After a 1 h incubation at 37°C, two additional volumes of serum-free medium and the appropriate amount of serum (as above) were added. This

medium was exchanged 48 h after transfection for a medium supplemented with 500 µg/ml neomycin (G418) to allow for selection of drug resistance. This selection medium was replaced every 3–4 days until distinct islands of surviving cells were visible. Individual clones of antibiotic-resistant cells were transferred to 24-well plates and grown in medium containing 200 µg/ml G418.

Immunoblotting of SNARE proteins

Preparation of protein extracts of PC12 membranes was performed as described previously [18]. Materials and methodology used for production and analysis of ECL-immunoblots were also essentially as described therein, with the following modifications: protein extracts in SDS sample buffer were incubated at 95°C for 5 min and subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on 5% (stacking) and 10% (separating) gels. After electrophoretic transfer of proteins to nitrocellulose, membranes were cut horizontally along appropriate molecular weight markers in order to separate regions harbouring proteins with apparent molecular weights of 10–20 kD, 21–29 kD and 30–50 kD. These membrane strips were incubated in one of the following monoclonal SNARE antibodies: syntaxin 1 antibody clone 78.3, synaptobrevin 2 antibody clone 69.1 (both obtained from Synaptic Systems, Göttingen, Germany) and SNAP-25 antibody MAB331 (purchased from Chemicon, Temecula, CA, USA).

Measurement of [³H]noradrenaline release

[³H]noradrenaline uptake in and superfusion of PC12 cell cultures were performed as described previously [23]. Culture discs with PC12 cells were incubated in 0.1 µM [³H]noradrenaline in culture medium supplemented with 1 mM ascorbic acid at 36°C for 1 h. After labelling, 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), hydroxyethylpiperazine ethanesulphonic acid (HEPES) (10), fumaric acid (0.5), N-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. Collection of 4-min superfusate fractions was started after a 60-min washout period to remove excess radioactivity.

Depolarization-dependent overflow of previously incorporated [³H]noradrenaline was triggered by inclusion of 30 or 100 mM KCl in the superfusion buffer during two 30-s periods 20 min apart (Fig. 2a). At the end of the experiments, the radioactivity remaining in the cells 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 2100 TR).

The spontaneous (unstimulated) rate of [³H] outflow was obtained by expressing the radioactivity of a collected fraction as percentage of the total radioactivity in the cultures at the beginning of the corresponding collection period. Values obtained during the 4-min period directly preceding the application of 30 mM K⁺ were used for the comparison of spontaneous (basal) tritium outflow in wild-type and pBoNT/C1 expressing PC12 cells. K⁺-evoked tritium overflow was calculated as the difference between the total [³H] outflow during and after stimulation and the estimated basal outflow which was assumed to decline linearly throughout the experiments. Therefore, basal outflow during periods of stimulation was assumed to equate the arithmetic mean of the samples preceding and those following stimulation, respectively. The difference between the total and the estimated basal outflow was expressed as a percentage of the total radioactivity in the cultures at the beginning of the respective stimulation (% of total activity).

Determination of cyclic AMP

The accumulation of cAMP in PC12 cell cultures was determined as described before [33]. After labelling of cellular purines with tritiated adenine (2.5 µCi/ml for 12 h), the medium was replaced by a buffer (120 mM NaCl, 3 mM KCl, 2 mM MgCl₂, 2 mM CaCl₂, 20 mM glucose, 10 mM HEPES, 10 mM LiCl, adjusted to pH 7.4 with NaOH) supplemented with 100 µM of the phosphodiesterase inhibitor Ro-20-1724 [4-(3-butoxy-4-methoxybenzyl)imidazolidin-2-one] and 1 U/ml adenosine deaminase. Dishes were then kept at room temperature for 105 min. During the last 15 min of this incubation period, the adenosine A_{2A} receptor agonist 2-p-(2-carboxyethyl)phenethylamino-5'-N-ethylcarboxamido-adenosine (CGS 21680), ADP (10 µM), apyrase (1 U/ml) or 100 mM KCl (NaCl was reduced accordingly) were also included in the medium. When appropriate, the P2Y₁₂ receptor antagonist 2MesAMP (100 µM; [14]) was present for the last 25 min. The incubation was terminated by exchanging the buffer for 1 ml of 2.5% perchloric acid containing 100 µM non-labelled cAMP followed by a 20-min incubation at 4°C. Subsequently, cAMP was separated from the other purines by a chromatographic procedure described before [33]. One tenth of each sample obtained as described above was used for the determination of the total radioactivity. The remaining 900 µl were neutralised by addition of 100 µl 4.2 M KOH and applied to Dowex 50 columns (AG 50W-X4; Bio-Rad, Vienna, Austria), which were then rinsed with 3 ml H₂O. The eluate obtained by the subsequent application of 8 ml H₂O was directly poured onto alumina

columns (Bio-Rad, Vienna, Austria), which were then washed with 6 ml H₂O. Finally, cAMP 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. The radioactivity in the fraction of cAMP was expressed as percentage of the total radioactivity incorporated in the cells.

Stimulation of PC12 cells, whether they were clonal cell lines or wild-type cells, with the adenosine A_{2A} receptor agonist CGS 21680 caused reproducible increases in the values of cAMP. However, the extent of basal and stimulated cAMP synthesis may vary between different preparations [33]. Hence, to be able to directly compare changes in CGS 21680-induced cAMP synthesis in different cell clones, values obtained in the presence of ADP, apyrase, 2MesAMP or KCl were expressed as percentage of the values obtained in the very same preparation, but in the absence of these agents (% of control).

Statistics

All data represent arithmetic means±SEM; *n* represents numbers of culture dishes. Statistical significance of differences between single data points was evaluated by the non-parametric Mann-Whitney test, and *p* values<0.05 were accepted as indicators of significance.

Results

To assess the role of vesicle exocytosis in the release of endogenous nucleotides from neuroendocrine cells, this study employed the autocrine/paracrine control of cAMP synthesis in PC12 cells via P2Y₁₂ receptors. To prevent

vesicle exocytosis, we generated cell clones stably expressing pBoNT/C1. Depending on the cell types being investigated, this toxin cleaves one or more SNARE proteins and thereby prevents vesicular transmitter release or hormone secretion [5].

Cleavage of SNARE proteins by the expression of the BoNT/C1 light chain

The major target of BoNT/C1 is syntaxin 1, which is cleaved by this toxin in all types of preparations [5]. Therefore, several pBoNT/C1 expressing clones were initially tested in immunoblots of harvested membranes for a lack of full-length syntaxin. Two of these pBoNT/C1 expressing clones (BT2 and BT7) were selected for further analysis.

In bovine chromaffin cells, BoNT/C1 was reported to cleave not only syntaxin 1, but also SNAP-25, even though the toxin failed to cleave purified and recombinant SNAP-25 [11]. To evaluate the efficiency of SNARE protein cleavage by the BoNT/C1 light chain expressed in PC12 cells, the immunostaining of syntaxin and SNAP-25 in Western blots was compared with that of synaptobrevin, which is undoubtedly insensitive to BoNT/C1 [11, 5]. As shown in Fig. 1, the stable expression of pBoNT/C1 markedly reduced or even abolished the staining of a protein with an apparent molecular weight of 37 kD by an anti-syntaxin antibody. In contrast, there were no clear-cut reductions in the staining of a 25-kD band by an anti-SNAP-25 antibody, and occasionally the immunostaining of this protein appeared to be even enhanced in pBoNT/C1 expressing clones (Fig. 1a). To quantify these changes in SNARE protein expression, the immunoblots were quantified by densitometry and the values obtained for the anti-syntaxin and anti-SNAP-25 immunoreactive bands were set

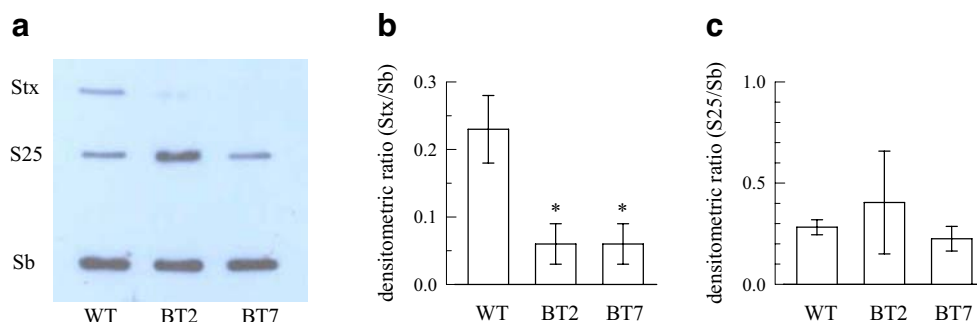


Fig. 1 Identification of SNARE proteins in membrane extracts from either wild-type (*WT*, untransfected) PC12 cells or from two PC12 cell clones stably expressing pBoNT/C1 (*BT2* and *BT7*). SDS-membrane extracts were separated on SDS-PAGE and transferred to nitrocellulose membranes. The membranes were cut horizontally to separate regions harbouring proteins with apparent molecular weights of 1–20 kD, 21–29 kD and 30–50 kD and immunostained with anti-syntaxin 1 antibody clone 78.3 (*Stx*), anti-SNAP-25 antibody MAB331 (*S25*)

and anti-synaptobrevin 2 antibody clone 69.1 (*Sb*), respectively. **a** Representative blot obtained as described above. **b** The relation between the anti-syntaxin and the anti-synaptobrevin immunoreactivities (*Stx/Sb*) as determined by densitometric analysis (*n*=3). **c** The relation between the anti-SNAP-25 and the anti-synaptobrevin immunoreactivities (*S25/Sb*) as determined by densitometric analysis (*n*=3). *Significant differences versus the results obtained with membranes from wild-type cells at *p*<0.05

in relation to those of the anti-synaptobrevin immunoreactive bands (Fig. 1b). This procedure revealed a significant reduction of syntaxin in the pBoNT/C1 expressing clones when compared with wild-type PC12 cells. SNAP-25 immunoreactivity, in contrast, was not significantly altered in these cell clones.

Inhibition of transmitter release by the expression of the BoNT/C1 light chain

Previously, it has been shown that the transient expression of pBoNT/C1 reduced stimulated vesicular catecholamine release from PC12 cells [10]. To evaluate whether catecholamine release was reduced in the pBoNT/C1 expressing clones, clonal and wild-type PC12 cells were labelled with [^3H]noradrenaline and the basal and depolarization-dependent outflow of radioactivity was determined. After washout of excess radioactivity during a 1-h washout period, all PC12 cell cultures steadily released small

amounts of radioactivity in the superfusion buffer (Fig. 2a). However, this spontaneous outflow of radioactivity was significantly less in the two pBoNT/C1 expressing clones than in untransfected PC12 cells (Fig. 2b).

In wild-type cells, the presence of 30 mM K^+ (Na^+ was reduced accordingly) for 30 s caused a reproducible increase in the outflow of radioactivity (Fig. 2a), but such an effect was hardly observed in the pBoNT/C1 expressing clones. Accordingly, the overflow of radioactivity triggered by 30 mM K^+ was significantly reduced in the pBoNT/C1 expressing clones in comparison with wild-type cells (Fig. 2c). This confirms that the expression of the BoNT/C1 light chain reduced spontaneous noradrenaline release and almost abolished release due to mild depolarizations.

Large intracellular Ca^{2+} increases have been reported to rescue transmitter release from cells poisoned with BoNT/C1 [7]. Therefore, PC12 cultures were also exposed to 100 mM K^+ (Na^+ was again reduced) for 30 s. Although this stimulation paradigm caused unequivocal increases in

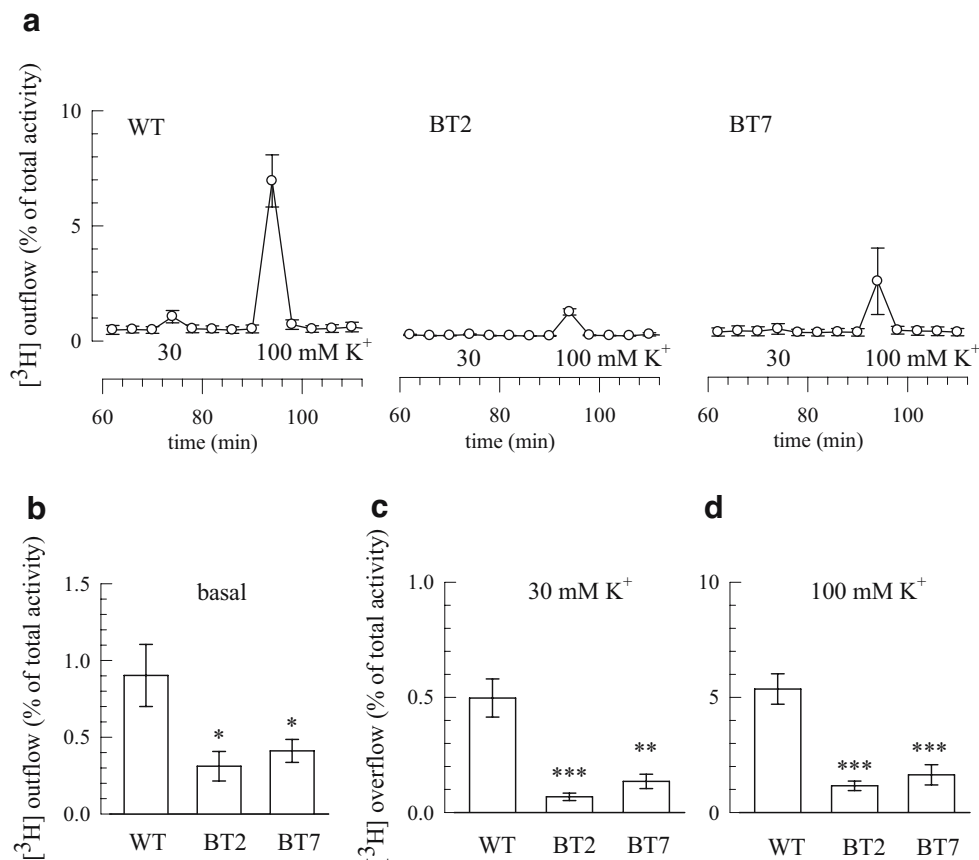


Fig. 2 Basal and depolarization-evoked [^3H]noradrenaline release from either wild-type (*WT*, untransfected) PC12 cells or from two PC12 cell clones stably expressing pBoNT/C1 (*BT2* and *BT7*). PC12 cell cultures were labelled with [^3H]noradrenaline and superfused. Subsequent to a 60-min washout period, 4-min fractions of superfusate were collected. Tritium overflow was stimulated twice (S1 after 72 min and S2 after 92 min of superfusion) by the presence of KCl (30 or 100 mM, each for 30 s). **a** Comparison of the time courses of

fractional [^3H] outflow from the three different kinds of cell cultures ($n=5$ to 6). **b** The amount of basal [^3H] outflow from the three PC12 cell cultures ($n=8$ to 9). **c** The amount of [^3H] overflow from the three PC12 cell cultures triggered by 30 mM KCl ($n=8$ to 9). **d** The amount of [^3H] overflow from the three PC12 cell cultures triggered by 100 mM KCl ($n=8$ to 9). *, **, ***Significant differences versus the results obtained with wild-type cells at $p<0.05$, $p<0.01$ and $p<0.001$, respectively

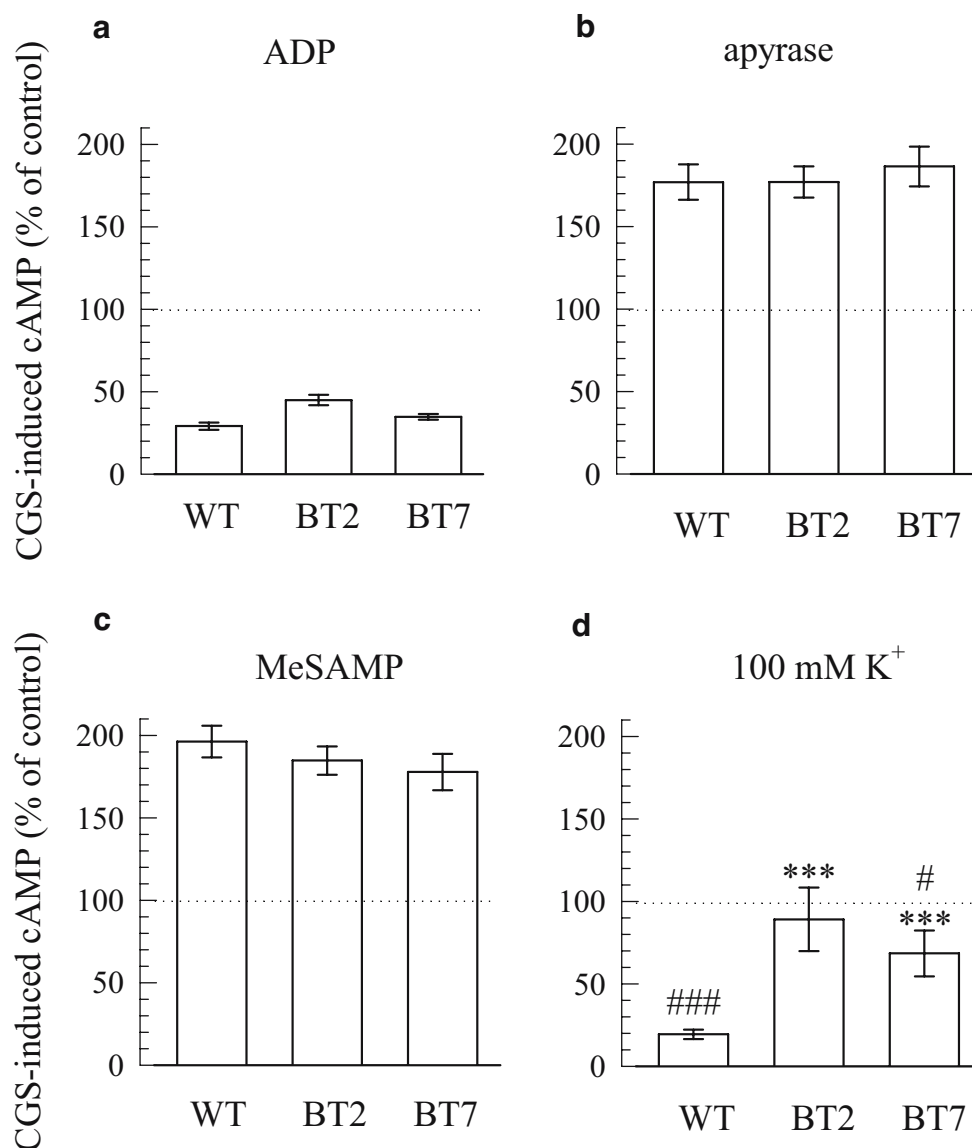


Fig. 3 Changes in cAMP synthesis caused by exogenous ADP or endogenously released nucleotides in either wild-type (*WT*, untransfected) PC12 cells or in two PC12 cell clones stably expressing pBoNT/C1 (*BT2* and *BT7*). After loading with [³H]adenine, PC12 cells were incubated in RO 20–1724 (100 μ M) for 105 min. During the last 15 min of this incubation period, 1 μ M CGS 21680 was present either alone or together with 10 μ M ADP (**a**), 1 U/ml apyrase (**b**), 100 μ M MeSAMP (**c**) or 100 mM KCl (**d**). The amount of radioactivity retrieved within the fraction of cyclic AMP (*cAMP*) was calculated as percentage of the total radioactivity extracted from the cell cultures. In order to compare the results of different cell cultures, values obtained in the presence of ADP, apyrase, 2MesAMP or KCl were expressed as

percentage of the values obtained in the very same preparation, but in the absence of these agents (% of control). **a** Results obtained in the presence of 10 μ M ADP are expressed as percentage of the results obtained in its absence (% of control; $n=6$ to 10). **b** Results obtained in the presence of 1 U/ml apyrase are expressed as percentage of the results obtained in its absence (% of control; $n=9$ to 10). **c** Results obtained in the presence of 100 μ M MeSAMP are expressed as percentage of the results obtained in its absence ($n=6$ to 9). **d** Results obtained in the presence of 100 mM KCl are expressed as percentage of the results obtained in its absence ($n=9$; *** $p<0.001$ versus the results obtained in wild-type cells; #, ### $p<0.05$ and $p<0.001$ versus the results obtained in the absence of KCl)

the outflow of radioactivity even from pBoNT/C1 expressing cells (Fig. 2a), the amount of tritium overflow was still reduced when compared with the values obtained in untransfected PC12 cells (Fig. 2d). Thus, the expression of the BoNT/C1 light chain efficiently reduced spontaneous as well as depolarization-evoked vesicular transmitter release.

Changes in the regulation of cAMP synthesis via P2Y receptors due to the expression of the BoNT/C1 light chain

In PC12 cells, activation of endogenous P2Y₁₂ receptors by the exposure to nucleotides causes an inhibition of adenylyl cyclase [33]. Furthermore, spontaneously released nucleo-

tides are also sufficient to partially activate these receptors, and depolarization of the cells fully activates the receptors through enhanced nucleotide release to maximally suppress cAMP synthesis [24]. In accordance with these previous data, exposure of wild-type PC12 cells to 10 μ M ADP significantly reduced the amount of cAMP generated due to the activation of A_{2A} adenosine receptor by 1 μ M CGS 21680 present for 15 min. In the two pBoNT/C1 expressing clones, the inhibition of cAMP accumulation was as pronounced as in the wild-type cells (Fig. 3a). Thus, the adenylyl cyclase inhibiting P2Y receptors were operating equally well in transfected as well as untransfected PC12 cells.

In the presence of apyrase (1 U/ml) to degrade extracellular nucleotides, the cAMP accumulation triggered by CGS 21680 was almost doubled. Again, this effect was not different between wild-type and pBoNT/C1 expressing cells (Fig. 3b). In accordance with these latter results, the blockade of the receptors by the P2Y₁₂ receptor antagonist 2-MeSAMP (100 μ M; [14]) also enhanced the stimulation of cAMP about twofold and there were no significant differences between the three types of PC12 cultures (Fig. 3b). Thus, the activation of the P2Y receptors negatively linked to adenylyl cyclase by spontaneously released nucleotides was the same whether pBoNT/C1 was expressed or not.

When wild-type PC12 cells were depolarized by 100 mM K⁺, the CGS 21680-induced cAMP accumulation was reduced to about the same extent as by 10 μ M ADP. However, in one (BT2) of the two pBoNT/C1 expressing clones, the depolarization of PC12 cells failed to cause any alteration in the CGS 21680-induced cAMP accumulation; and in the other clone (BT7), this inhibition was significantly weaker when compared with the values obtained in wild-type PC12 cells (Fig. 3d). Thus, depolarization-dependent inhibition of the cAMP synthesis via P2Y₁₂ receptors was greatly impaired in the pBoNT/C1 expressing cells.

Discussion

In non-neural cells, release of nucleotides is mostly non-vesicular and involves, for instance, ATP binding cassette transporters, stretch-activated cation channels or connexin hemichannels [22]. In neurons, however, nucleotide release is generally believed to rely on vesicle exocytosis [27]. Here, we employed the autocrine/paracrine activation of P2Y₁₂ receptors in PC12 cells to investigate whether nucleotide release from neuroendocrine cells might involve a non-vesicular component.

Vesicle exocytosis involves the SNARE proteins syntaxin, SNAP-25 and synaptobrevin, and cleavage of one or more of these proteins by clostridial neurotoxins reduces or even

abolishes exocytotic transmitter release [5]. The primary target of BoNT/C1 is syntaxin, and its inhibitory effect on transmitter release has been correlated with the cleavage of this protein [3]. However, in cultures of bovine chromaffin cells [11], spinal cord [37] or hippocampal neurons [7], BoNT/C1 was reported to cleave not only syntaxin, but also SNAP-25. In contrast, in sensory neurons the toxin was reported to act exclusively on syntaxin [31]. Furthermore, in cell-free assays BoNT/C1 was found to leave either native SNAP-25 after subcellular fractionation or recombinant SNAP-25 unaltered [11, 37]. Thus, the effects of BoNT/C1 on SNARE proteins appear to vary between different preparations. In this study, expression of the BoNT/C1 light chain reduced syntaxin, but not SNAP-25 immunoreactivity detected in PC12 cells. Likewise, in the insulin secreting β -cell line HIT-T15 expression of the BoNT/C1 light chain only affected syntaxin, even though application of recombinant toxin to permeabilized cells did lead to a reduction in SNAP-25 [20]. Hence, heterologous expression of the toxin's light chain in neuroendocrine cells does not entirely reproduce the effects of toxin application to these cells. Syntaxin was reported to be more sensitive to BoNT/C1 light chain than SNAP-25 [20] and a 1000-fold higher light chain concentration of BoNT/C was required for SNAP-25 cleavage when compared with the other SNAP-25-targeting serotypes BoNT/A or BoNT/E [34]. Thus, the resistance of SNAP-25 to heterologously expressed BoNT/C1 light chain is likely due to the relatively low toxin affinity and insufficient expression levels of the peptide.

Nevertheless, in functional experiments, expression of the BoNT/C1 light chain significantly reduced spontaneous as well as depolarization-evoked release of [³H]noradrenaline. On the one hand, this confirms that the cleavage of syntaxin alone is sufficient to reduce transmitter release [3]. On the other hand, this result corroborates that catecholamines are released from PC12 cells through vesicular mechanisms and these are largely impaired by the expression of the BoNT/C1 light chain [10].

Considering that the heterologous expression of the BoNT/C1 light chain did efficiently reduce vesicle exocytosis, the question arises as to how this manipulation altered the release of endogenous nucleotides. In PC12 cells, endogenously released nucleotides activate P2Y₁₂ receptors, which mediate a decrease in the synthesis of cAMP triggered by the activation of A_{2A} adenosine receptors [24]. In accordance with these previous results, exposure of non-transfected PC12 cells to the P2Y₁₂ receptor antagonist 2-MeSAMP enhanced the cAMP synthesis. An equivalent effect was achieved when the cells were incubated in the nucleotide degrading enzyme apyrase, thus indicating that spontaneously released nucleotides continuously activated the P2Y₁₂ receptors.

To be able to evaluate whether the generation of PC12 clones stably expressing the BoNT/C1 light chain might interfere with the above autocrine/paracrine signalling loop only by affecting exocytosis, we had to establish that the function of the P2Y₁₂ receptor was not compromised by the toxin's light chain or by the clonal selection procedure. Accordingly, 10 μM ADP, a saturating concentration of the nucleotide in the inhibition of cAMP synthesis in PC12 cells [33], were applied to non-transfected cells and to the two chosen cell clones (BT2 and BT7): the inhibition of cAMP accumulation was not different in the three cultures. Thus, the control of cAMP synthesis via the P2Y₁₂ receptor was not altered by pBoNT/C1 expression or by clonal selection. Previously, we had found that the control of voltage-gated Ca²⁺ channels via this receptor subtype was the same in a number of PC12 cell clones expressing various types of membrane receptors [19].

Knowing that the P2Y₁₂ receptors operated independently of the BoNT/C1 light chain, the autocrine/paracrine activation of the receptors was assessed. In the toxin expressing clones, the degradation of extracellular nucleotides by apyrase and the blockade of P2Y₁₂ receptors by 2-MesAMP had identical effects on cAMP accumulation as in non-transfected cells. This demonstrates that the spontaneous release of nucleotides was not significantly affected by the BoNT/C1 light chain.

Enhancement of nucleotide release by depolarization of PC12 cells fully activates P2Y₁₂ receptors and thereby reduces cAMP synthesis to about the same extent as 10 μM ADP. This phenomenon is based on Ca²⁺-dependent vesicle exocytosis as it is suppressed when Ca²⁺ influx is prevented by the blockade of voltage-gated Ca²⁺ channels or when extracellular nucleotides are degraded by apyrase [24]. In support of this conclusion, the inhibition of cAMP synthesis through a depolarization of PC12 cells by 100 mM K⁺ was either significantly attenuated or entirely abolished in the pBoNT/C1 expressing clones. Hence, the K⁺-evoked and thus Ca²⁺-dependent release of nucleotides was largely reduced by the BoNT/C1 light chain.

Taken together, the present results show that the expression of the BoNT/C1 light chain impairs vesicular transmitter release from PC12 cells, but does not alter the autocrine/paracrine activation of P2Y receptors by spontaneously released nucleotides. Thus, the spontaneous nucleotide release from neuroendocrine cells may occur independently of vesicle exocytosis. The depolarization-evoked nucleotide release, in contrast, relies predominantly on exocytotic mechanisms.

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References

1. Abbracchio MP, Burnstock G, Boeynaems JM, Barnard EA, Boyer JL, Kennedy C, Knight GE, Fumagalli M, Gachet C, Jacobson KA, Weisman GA (2006) International Union of Pharmacology LVIII: update on the P2Y G protein-coupled nucleotide receptors: from molecular mechanisms and pathophysiology to therapy. *Pharmacol Rev* 58:281–341
2. Anderson CM, Parkinson FE (1997) Potential signalling roles for UTP and UDP: sources, regulation and release of uracil nucleotides. *Trends Pharmacol Sci* 18:387–392
3. Blasi J, Chapman ER, Yamasaki S, Binz T, Niemann H, Jahn R (1993) Botulinum neurotoxin C1 blocks neurotransmitter release by means of cleaving HPC-1/syntaxin. *EMBO J* 12:4821–4828
4. Boehm S, Kubista H (2002) Fine tuning of sympathetic transmitter release via ionotropic and metabotropic presynaptic receptors. *Pharmacol Rev* 54:43–99
5. Burgoyne RD, Morgan A (2003) Secretory granule exocytosis. *Physiol Rev* 83:581–632
6. Burnstock G, Campbell G, Satchell D, Smythe A (1970) Evidence that adenosine triphosphate or a related nucleotide is the transmitter substance released by non-adrenergic inhibitory nerves in the gut. *Br J Pharmacol* 40:668–688
7. Capogna M, McKinney RA, O'Connor V, Gähwiler BH, Thompson SM (1997) Ca²⁺ or Sr²⁺ partially rescues synaptic transmission in hippocampal cultures treated with botulinum toxin A and C, but not tetanus toxin. *J Neurosci* 17:7190–7202
8. Coco S, Calegari F, Pravettoni E, Pozzi D, Taverna E, Rosa P, Matteoli M, Verderio C (2003) Storage and release of ATP from astrocytes in culture. *J Biol Chem* 278:1354–1362
9. Currie KPM, Fox AP (1996) ATP serves as a negative feedback inhibitor of voltage-gated Ca²⁺ channel currents in bovine adrenal chromaffin cells. *Neuron* 16:1027–1036
10. Fisher RJ, Burgoyne RD (1999) The effect of transfection with Botulinum neurotoxin C1 light chain on exocytosis measured in cell populations and by single-cell amperometry in PC12 cells. *Pflugers Arch* 437:754–762
11. Foran P, Lawrence GW, Shone CC, Foster KA, Dolly JO (1996) Botulinum neurotoxin C1 cleaves both syntaxin and SNAP-25 in intact and permeabilized chromaffin cells: correlation with its blockade of catecholamine release. *Biochemistry* 35:2630–2636
12. Gerasimovska EV, Ahmad S, White CW, Jones PL, Carpenter TC, Stenmark KR (2002) Extracellular ATP is an autocrine/paracrine regulator of hypoxia-induced adventitial fibroblast growth. *J Biol Chem* 277:44638–44650
13. Hollins B, Ikeda SR (1997) Heterologous expression of a P2x-purinoreceptor in rat chromaffin cells detects vesicular ATP release. *J Neurophysiol* 78:3069–3076
14. Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, Yang RB, Nurden P, Nurden A, Julius D, Conley PB (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature* 409:202–207
15. Holton P (1959) The liberation of adenosine triphosphate on antidromic stimulation of sensory nerves. *J Physiol* 145:494–504
16. Junankar PR, Karjalainen A, Kirk K (2002) The role of P2Y1 purinergic receptors and cytosolic Ca²⁺ in hypotonically activated osmolyte efflux from a rat hepatoma cell line. *J Biol Chem* 277:40324–40334
17. Knight GE, Bodin P, De Groat WC, Burnstock G (2002) ATP is released from guinea pig ureter epithelium on distension. *Am J Physiol Renal Physiol* 282:F281–F288
18. Kubista H, Edelbauer H, Boehm S (2004) Evidence for structural and functional diversity among SDS-resistant SNARE complexes in neuroendocrine cells. *J Cell Sci* 117:955–966

19. Kudlacek O, Just H, Korkhov VM, Vartian N, Klinger M, Pankevych H, Yang Q, Nanoff C, Freissmuth M, Boehm S (2003) The human D2 dopamine receptor synergizes with the A2A adenosine receptor to stimulate adenylyl cyclase in PC12 cells. *Neuropsychopharmacology* 28:1317–1327
20. Lang J, Zhang H, Vaidyanathan VV, Sadoul K, Niemann H, Wollheim CB (1997) Transient expression of botulinum neurotoxin C1 light chain differentially inhibits calcium and glucose induced insulin secretion in clonal beta-cells. *FEBS Lett* 419:13–17
21. Lazarowski ER, Boucher RC, Harden TK (2000) Constitutive release of ATP and evidence for major contribution of ecto-nucleotide pyrophosphatase and nucleoside diphosphokinase to extracellular nucleotide concentrations. *J Biol Chem* 275:31061–31068
22. Lazarowski ER, Boucher RC, Harden TK (2003) Mechanisms of release of nucleotides and integration of their action as P2X- and P2Y-receptor activating molecules. *Mol Pharmacol* 64:785–795
23. Lechner SG, Dorostkar MM, Mayer M, Edelbauer H, Pankevych H, Boehm S (2004) Autoinhibition of transmitter release from PC12 cells and sympathetic neurons through a P2Y receptor-mediated inhibition of voltage-gated Ca^{2+} channels. *Eur J Neurosci* 20:2917–2928
24. Moskvina E, Unterberger U, Boehm S (2003) Activity-dependent autocrine-paracrine activation of neuronal P2Y receptors. *J Neurosci* 23:7479–7488
25. North RA (2002) Molecular physiology of P2X receptors. *Physiol Rev* 82:1013–1067
26. Ostrom RS, Gregorian C, Insel PA (2000) Cellular release of and response to ATP as key determinants of the set-point of signal transduction pathways. *J Biol Chem* 275:11735–11739
27. Pankratov Y, Lalo U, Verkhratsky A, North RA (2006) Vesicular release of ATP at central synapses. *Pflugers Arch* 452:589–597
28. Schlosser SF, Burgstahler AD, Nathanson MH (1996) Isolated rat hepatocytes can signal to other hepatocytes and bile duct cells by release of nucleotides. *Proc Natl Acad Sci U S A* 93:9948–9953
29. Schneggenburger R, Neher E (2005) Presynaptic calcium and control of vesicle fusion. *Curr Opin Neurobiol* 15:266–274
30. Schwiebert EM, Wallace DP, Braunstein GM, King SR, Peti-Peterdi J, Hanaoka K, Guggino WB, Guay-Woodford LM, Bell BD, Sullivan LP, Grantham JJ, Taylor AL (2002) Autocrine extracellular purinergic signaling in epithelial cells derived from polycystic kidneys. *Am J Physiol Renal Physiol* 282: F763–F775
31. Stanley EF, Mirotnik RR (1997) Cleavage of syntaxin prevents G-protein regulation of presynaptic calcium channels. *Nature* 385:340–343
32. Torres B, Zamboni AC, Insel PA (2002) P2Y₁₁ receptors activate adenylyl cyclase and contribute to nucleotide-promoted cAMP formation in MDCK-D₁ cells. *J Biol Chem* 277:7761–7765
33. Unterberger U, Moskvina E, Scholze T, Freissmuth M, Boehm S (2002) Inhibition of adenylyl cyclase by neuronal P2Y receptors. *Br J Pharmacol* 135:673–684
34. Vaidyanathan VV, Yoshino K, Jahnz M, Dörries C, Bade S, Nauenburg S, Niemann H, Binz T (1999) Proteolysis of SNAP-25 isoforms by botulinum neurotoxin types A, C, and E: domains of amino acid residues controlling the formation of enzyme-substrate complexes and cleavage. *J Neurochem* 72:327–337
35. von Kugelgen I, Allgaier C, Schober A, Starke K (1994) Co-release of noradrenaline and ATP from cultured sympathetic neurons. *Neuroscience* 61:199–202
36. Wang Y, Roman R, Lidofsky SD, Fitz JG (1996) Autocrine signalling through ATP release represents a novel mechanism for cell volume regulation. *Proc Natl Acad Sci U S A* 93:12020–12025
37. Williamson LC, Halpern JL, Montecucco C, Brown JE, Neale EA (1996) Clostridial neurotoxins and substrate proteolysis in intact neurons: botulinum neurotoxin C acts on synaptosomal-associated protein of 25 kDa. *J Biol Chem* 271:7694–7699
38. You J, Jacobs CR, Steinberg TH, Donahue HJ (2002) P2Y purinoceptors are responsible for oscillatory fluid flow-induced intracellular Ca^{2+} mobilization in osteoblastic cells. *J Biol Chem* 277:48724–48729