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INTERACTION BETWEEN ATP AND NERVE GROWTH FACTOR SIGNALLING IN THE SURVIVAL AND NEURITIC OUTGROWTH FROM PC12 CELLS

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Abstract—In a previous study we used P2 receptor antagonists to inhibit diverse responses that nerve growth factor (NGF) promotes and coordinates in PC12 cells and we suggested that P2 receptors partake in the NGF signalling cascade. In this paper, we examine the direct role of extracellular P2 receptor agonists as neurotrophic factors. ATP and 2-Cl-ATP promote neurite regeneration after priming PC12 cells with NGF and the effect is dose-dependent, with an EC_{50} of about 5 and 3 μM, respectively. The number of cell clumps bearing neurites was maximally induced in day 1 and it was maintained up to about one week by ATP, or up to at least 2 weeks by 2-Cl-ATP. The involvement of P1 receptors or intracellular inosine in these actions was excluded, whereas various antagonists of P2 receptors were inhibitory. Moreover, NGF and ATP caused a direct up-regulation of $P2X_2$, $P2X_3$, $P2X_4$ and $P2Y_2$, but not $P2Y_4$ receptor proteins under neurite-regenerating conditions, as well as extracellular signal-regulated kinase (Erk)1-2 tyrosine/threonine phosphorylation and activation. Finally, ATP, 2-Cl-ATP and ATPγS enhanced neurite initiation evoked by sub-optimal NGF concentrations and ATP and 2-Cl-ATP fully sustained survival of PC12 cells after serum deprivation.

Our results establish that P2 receptor agonists can behave as neurotrophic factors for neuronal cells and suggest a potential interplay between ATP and NGF in the signalling pathways triggered on their target cells. © 2001 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: P2X, P2Y, extracellular signal-regulated kinases, neurite regeneration, cell death.

The well-established role of purines as neurotransmitters and neurotrophic factors is mediated by the activation of specific extracellular receptors (Neary et al., 1996; Burnstock, 1997; Ralevic and Burnstock, 1998; Fields and Stevens, 2000). These are P1 receptors for adenosine (A1, A2A, A2B and A3 subtypes, all G-protein-coupled) and P2 receptors for ATP (P2X ionotropic and P2Y metabotropic) (Abbracchio and Burnstock, 1994; Burnstock and King, 1996). Whereas the neuroprotective properties of A1 adenosine receptor analogues have been known for several years, the role of other adenosine receptor subtypes is highly controversial, since both protective and detrimental actions have been reported (Abbracchio et al., 1998; Liang and Jacobson, 1998; Macek et al., 1998; Ongini and Schubert, 1998). Similarly, P2 receptor effects in the modulation of brain damage still awaits further characterisation, although recent data from CNS primary cultures would suggest synergism with glutamate-induced excitotoxicity and with apoptotic pathways (Volonté and Merlo, 1996; Volonté et al., 1999; Cavaliere et al., 2001a,b).

In PC12 cells, both P2X and P2Y receptors have been described (Kim and Rabin, 1994; Michel et al., 1996; Murayama et al., 1998; Swanson et al., 1998; Arslan et al., 2000). Furthermore, ATP released by these cells (Zimmermann, 1996; Gusovsky et al., 1988) induces neurotransmitter release, intracellular calcium modulation and second messenger activation (Sela et al., 1991; Choi and Kim, 1996; Khiroug et al., 1997; Chen and Sun, 1998; Soltoff, 1998; Soltoff et al., 1998). Our work with PC12 cells has shown that nerve growth factor (NGF)-dependent initiation and stability of neurites, as well as extracellular and intracellular protein phosphorylation, become impaired by P2 receptor phar-

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Abbreviations: AMPPNP, 5′-adenylyl-β,γ-imidodiphosphate; AP4A, P^1,P^4-di(adenosine-5′)tetraphosphate; β,γmet-ATP, β,γ-methylene-ATP; 8-Br-ATP, 8-bromo-ATP; BzATP, benzoyl–benzoyl-ATP; DIDS, 4,4′-diisothiocyanatostilbene-2, 2′disulphonic acid; ERK, extracellular signal-regulated kinase; 2-MeSATP, 2-methylthio-ATP; NBT, nitrobenzyl-6-thio-inosine; NGF, nerve growth factor; *o*-ATP, ATP-2′,3′-dialdehyde; P, phosphorylated; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PIT, 2,2′-pyridylisatogen; P5P, pyridoxal-5′-phosphate; PPADS, pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid 4-sodium; SDS, sodium dodecyl sulphate.

macological inhibition. However, cellular duplication, 5′-nucleotidase activity or NGF-induced tyrosine autophosphorylation of TrkA receptor are not affected (D'Ambrosi et al., 2000).

The aim of the present study is now to examine whether there is a direct function for extracellular P2 receptor agonists as neurotrophic agents, particularly in sustaining neurite regeneration and survival of PC12 neuronal cells.

EXPERIMENTAL PROCEDURES

Cell culture

PC12 cells were cultured on collagen-coated culture dishes in RPMI 1640 medium (plus 50 U/ml of penicillin and 50 mg/ml streptomycin) supplemented with 10% heat inactivated horse serum and 5% foetal bovine serum (Greene and Tischler, 1976). Mouse submaxillary NGF (Mobley et al., 1972) was directly added to the cultures from a stock of 100 μg/ml.

Neurite regeneration studies

PC12 cells were pre-treated with NGF for 1–2 weeks and then cultured for regeneration on collagen-coated tissue culture dishes, in RPMI 1640 medium, in the presence or absence of NGF (50 ng/ml), and with or without various P2 receptor agonists and antagonists (Sigma, Milan, Italy), as specified. Cultures were scored at different times, for proportion of neurite-bearing cell clumps, as described (Burstein and Greene, 1978). Triplicate wells were scored and counts represent means ± S.E.M.

Survival studies

For serum-free experiments, cells in stock cultures were washed several times with serum-free RPMI 1640 medium. They were detached by repeated triturating and washed again in serum-free medium by several cycles of centrifugation/resuspension. The cells were kept in serum-free medium and, at different time thereafter, they were lysed with a detergent-containing solution, which provides a uniform suspension of single, intact viable nuclei. The latter were quantified in a hemocytometer. Broken or damaged nuclei were not included in the counts. In all cases, triplicate wells were scored and counts represent means ± S.E.M.

Cell protein extraction

PC12 cells were harvested with ice-cold RIPA buffer: phosphate-buffered saline (PBS), 1% Nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), plus 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 μg/ml leupeptin, 10 mM NaF, 1 mM Na_3VO_4, 5 μM $ZnCl_2$. Cellular lysates were kept for 30 min on ice and then centrifuged for 10 min at $10\,000 \times g$, at 4°C. Supernatants were collected and assayed for protein quantification.

SDS–polyacrylamide gel electrophoresis (PAGE), western blotting and immunoreactions

Analysis of specific protein components was performed by SDS–PAGE on 10% polyacrylamide gels, as described (Laemmli, 1970), loading the same amount of total protein for each well. Total proteins were transferred on polyvinylidene difluoride (PVDF) membrane and blots were probed for 2 h at room temperature with the specified antiserum or antibodies, followed by horseradish peroxidase-coupled secondary antibody and analysed using enhanced chemiluminescence (ECL). Anti-$P2X_2$, $P2X_4$ (Alomone Labs., Jerusalem, Israel), anti-extracellular signal-regulated kinase (ERK) (Calbiochem, Germany), phosphorylated (P)-Erk (Santa Cruz, Biotechnology, Santa Cruz, CA, USA) were used at 1:500. Anti-$P2X_3$ (Neuromics, Minneapolis, MN, USA) was used at 1:1000, anti-$P2Y_{2,4}$ (Alomone Labs., Jerusalem, Israel) were used at 1:200 and 1:300, respectively. Band detection and quantification was performed by Kodak Image Station 440CF.

Protein determination

Protein concentrations were determined by the method of Bradford (Bradford, 1976), using reagents and protocols purchased from Bio-Rad Laboratories (Richmond, CA, USA) and with bovine serum albumin as a standard.

RESULTS

Selected P2 receptor agonists promote neurite regeneration in PC12 cells

NGF can prime PC12 cells for neurite regeneration (Burstein and Greene, 1978) that, in contrast to neurite initiation, has a short time lag, is extended to the greater part of the cell population within 48 h and is not blocked by transcription inhibitors. Under these conditions, we have shown here that extracellular ATP, dose dependently, with EC_{50} at about 5 μM and maximal effect at 100 μM, promoted and sustained neurite regeneration for about 1 week, in a way morphologically similar to that obtained with optimal concentrations of NGF (Figs. 1 and 2). The neuritogenic action started within a few hours after exposing PC12 cells to ATP (data not shown) and it was maximally achieved in 1 day, when long, branching neurites were regenerated (Figs. 1 and 2). As observed by phase-contrast microscopy after 24 h, the simultaneous addition of NGF (used at sub-optimal concentrations) and ATP further augmented cell–cell adhesion, length, number, thickness and fasciculation of neurites (Fig. 1D, F). The effect was reversible (data not shown) and ATP was never toxic (up to 3 mM) for PC12 cells, under the experimental conditions adopted. Additional agonists at P2 receptor were tested for their potential effect on neurite regeneration. The compounds 5′-adenylyl-β,γ-imidodiphosphate (AMPPNP), β,γ-methylene-ATP (β,γmet-ATP, Fig. 2A), benzoyl–benzoyl-ATP (BzATP), 8-bromo-ATP (8-Br-ATP) (all up to 100 μM, data not shown), P^1,P^4-di(adenosine-5′)tetraphosphate (AP4A) (up to 30 μM, data not shown) were ineffective and 2-methylthio-ATP (2-MeSATP) or ADP (up to 100 μM) elicited only mild actions (30–50% over control, data not shown). In contrast, neuritogenesis was transiently sustained for up to about 1 week by ATPγS (Fig. 2A) and for at least 2 weeks (data not shown) by 2-Cl-ATP (Fig. 2). The latter, with EC_{50} at about 3 μM (Fig. 2B), induced shorter and less branched, but stable and thicker processes than NGF itself. Moreover, ATP, ATPγS and 2-Cl-ATP strongly augmented neurite initiation promoted by sub-optimal NGF concentrations, whereas BzATP and 8-Br-ATP produced only a moderate effect in this regard (Fig. 3A). Under both

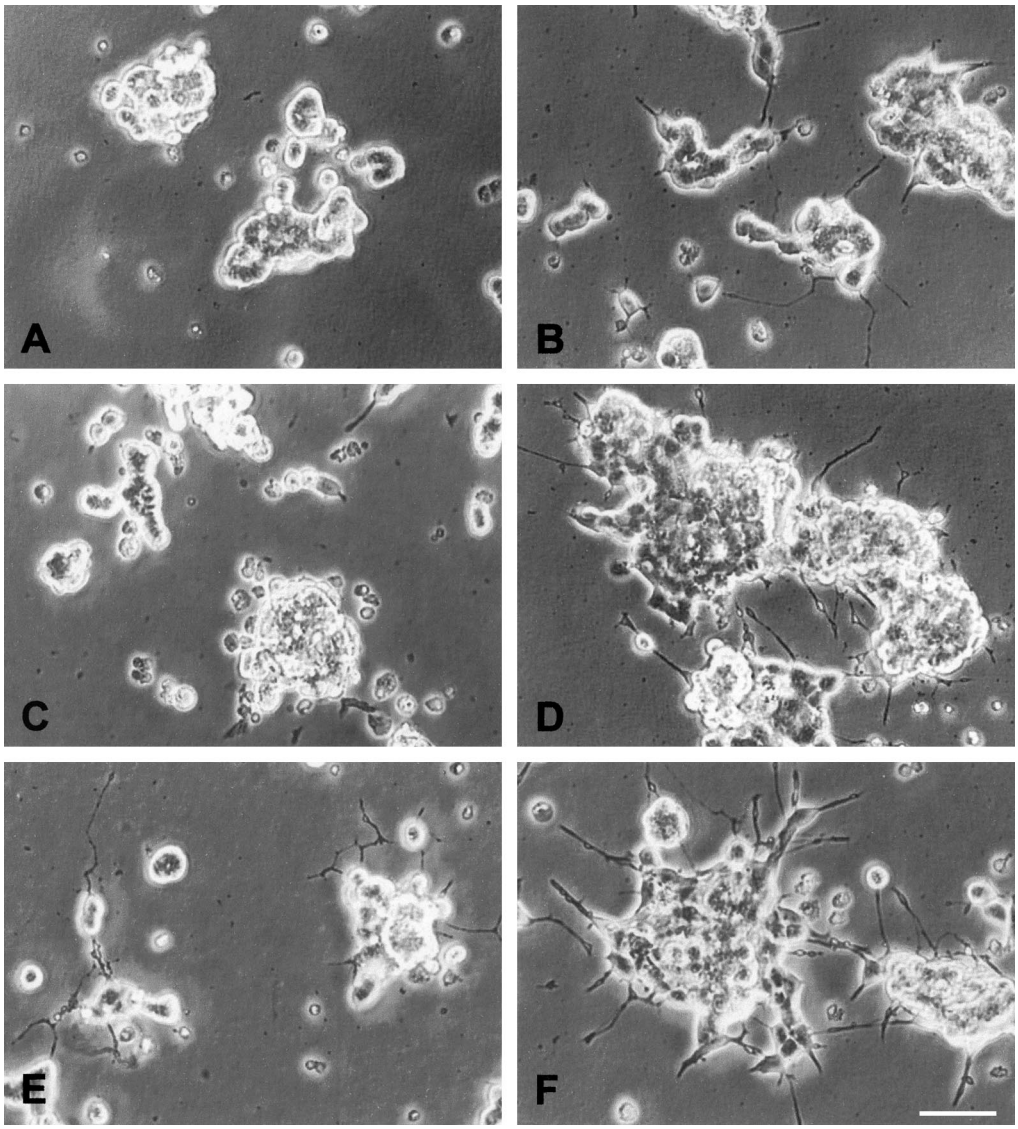

Fig. 1. Extracellular ATP induces neurite regeneration. PC12 cells were primed with 50 ng/ml NGF for 2 weeks, mechanically shorn of neurites and replated in the absence (A, B) or in the presence of NGF at 5 ng/ml (C, D) or 50 ng/ml (E, F), in the simultaneous presence of 100 μM ATP (B, D, F). Phase-contrast photomicrographs were taken after 24 h. Scale bar = 40 μm.

neurite-regenerating (Fig. 4) and -potentiating (Fig. 3B) conditions, the P2 receptor antagonist reactive blue 2 (10 μM) prevented ATP and 2-Cl-ATP effects, whereas pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid 4-sodium (PPADS) (30 μM) inhibited mostly ATP actions (Figs. 3B and 4). Additional P2 receptor antagonists were barely effective (Figs. 3 and 4). Adenosine (up to 200 μM) and inosine (up to 100 μM) did not sustain neurite regeneration (Fig. 5A), whereas caffeine did not significantly prevent it (data not shown). ARL67156 (100 μM), blocking ecto-ATPases, improved the effect of sub-optimal ATP concentrations (Fig. 5A), whereas nitrobenzyl-6-thio-inosine (NBT; 20 μM), inhibiting purine nucleoside transport, did not block ATP-evoked neuritogenesis (Fig. 5B).

ATP up-regulates P2X$_{2,3,4}$, and P2Y$_2$ proteins and it activates Erk1-2 kinases

We have previously reported that NGF directly stimulated the expression of $P2X_2$ receptor in PC12 cells (D'Ambrosi et al., 2000). We have now shown that under neurite-regenerating conditions ATP and NGF up-regulated the protein expression of $P2X_2$ (+67%, +77% respectively, maximal effect in 48 h), $P2X_3$ (+41%, +34% respectively, in 24 h), $P2X_4$ (+108%, +142% respectively, in 48 h) and $P2Y_2$ (+63%, +22% respectively, in 24 h), but not $P2Y_4$ (Fig. 6A). This expression was further augmented by the simultaneous addition of both factors and the neuritogenic-ineffective β,γmet-ATP failed to induce similar actions (Fig. 6A). Under neurite-regenerating conditions, ATP or NGF modulated not only P2 receptor expression, but also intracellular tyrosine phosphorylation and activation of Erk1-2 kinases. These were increased about 6- or 5-fold after 5 min with NGF or ATP, respectively. Whereas NGF induction remained constant for 30 min at least, ATP-evoked stimulation was instead transient, decreasing after 5 min of treatment (Fig. 6B). This endurance difference might very well explain the stable effect of NGF on neurite regeneration, with respect to the transient ATP action.

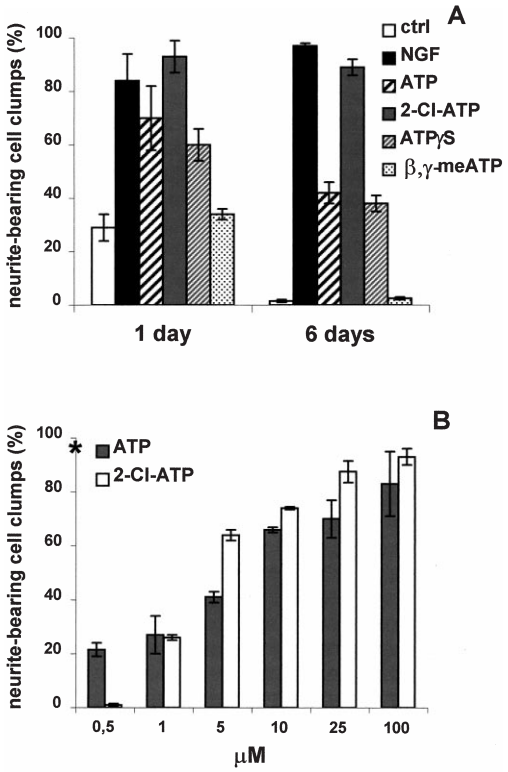

Fig. 2. P2 receptor agonists promote neurite regeneration in a time- and dose-dependent manner. PC12 cells were primed with 50 ng/ml NGF for 2 weeks, shorn of neurites and replated for regeneration with: (A) 50 ng/ml NGF, or ATP, 2-Cl-ATP, ATPγS, β,γmet-ATP, all used at 100 μM; (B) ATP and 2-Cl-ATP at different concentration, as indicated. Neurite-bearing cell clumps were scored after 1, 6 days (A) or after about 60 h (B). Counts represent means ± S.E.M. ($n = 3$). *Represents NGF value, reported as 100% positive control. Ctrl, control.

P2 receptor agonists sustain survival of PC12 cells after serum deprivation

It is well established that NGF-evoked neurite outgrowth and survival lie on different mechanistic pathways (Klesse et al., 1999; Kaplan and Miller, 2000). We have therefore tested also this parameter to characterise the biological effects of ATP as a neurotrophic agent. By direct count of intact viable nuclei, we have shown that extracellular ATP maintained PC12 cells alive for at least 1 week after serum deprivation. When inspected by phase-contrast microscopy under these conditions, the cells exhibited a fully preserved healthy morphological appearance, brightness and integrity of cell bodies. Nevertheless, they did not elicit a distinctly flattened appearance, short cytoplasm extensions or neurites (Fig. 7). Among additional P2 receptor agonists, ATPγS, BzATP, AP4A and 2-MeSATP elicited a 2-fold transient (up to 24 h) survival effect (data not shown), whereas 2-Cl-ATP sustained durable cell viability (Fig. 8) and short neurites for at least 14 days after treatment (data not shown). Only a few P2 receptor antagonists partially impaired survival sustained by either extracellular ATP or 2-Cl-ATP (Fig. 8), whereas the P1 receptor antagonist caffeine was ineffective (data not shown).

DISCUSSION

Trophic functions for extracellular purine molecules have been extensively described for both neuronal and non-neuronal cells (Neary et al., 1996; Burnstock, 1997). It is well known that analogues of cAMP enhance (Gunning et al., 1981; Herman et al., 1994) or inhibit (Lillien and Claude, 1985; Herman et al., 1994) growth factor-mediated neurite outgrowth; cGMP promotes neuritogenesis in hippocampal neurones (Hindley et al., 1997) and inosine stimulates morphological differentiation of sympathetic and retinal cells (Zurn and Do, 1988; Benowitz et al., 1998; Petrausch et al., 2000). Whereas these effects occur without interaction with specific cell-surface receptors, some purines can elicit neurotrophic actions mediated by purinoceptors. For example, adenosine derivatives acting on P1 receptors enhance neuritogenesis in sympathetic and sensory ganglia, induce PC12 cells to extend short neurites (Gysbers et al., 2000) and elicit a robust synergistic effect with NGF (Guroff et al., 1981; Braumann et al., 1986; Gysbers et al., 2000). However, there are no direct indications about P2 receptor involvement in neuritogenesis. It is known that ATP stimulates in PC12 cells intracellular pathways such as mitogen-activated protein kinases, calcium-activated tyrosine kinase Pyk2 (Swanson et al., 1998), related adhesion focal tyrosine kinase, protein kinase C (Soltoff

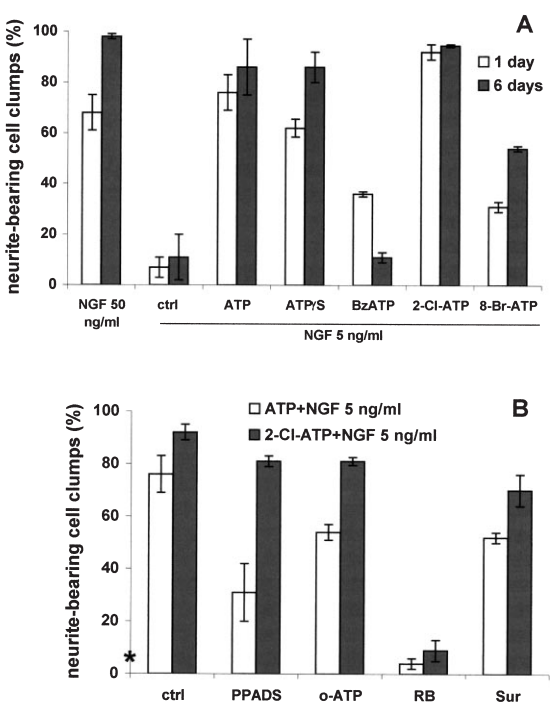

Fig. 3. Effect of P2 receptor agonists and antagonists on NGF-dependent neurite initiation. PC12 cells were plated with P2 receptor agonists ATP, 2-Cl-ATP (A, B) ATPγS, BzATP and 8-Br-ATP (A), all at 100 μM, in the simultaneous presence of 5 ng/ml NGF (A, B), or with NGF alone at 50 ng/ml (A). P2 receptor antagonists were 30 μM PPADS, 50 μM ATP-2′,3′-dialdehyde (*o*-ATP), 10 μM reactive blue 2 (RB), 100 μM suramin (Sur) (B). Cells were scored for proportion of neurite-bearing cells clumps after 1 (A, B) or 6 days (A). Counts represent means ± S.E.M. ($n = 3$). (B) *Stands for 5 ng/ml NGF value at 24 h. Ctrl, control.

et al., 1998) and Ca^{2+} transients (Arslan et al., 2000), but without neuritogenic activity. Nevertheless, the hypothesis of P2 receptor involvement in neuronal differentiation was indirectly formulated using P2 receptor antagonists (D'Ambrosi et al., 2000). In the present work, we substantiate this hypothesis with new experimental data and establish a direct dual effect of extracellular P2 receptor agonists as neurotrophic agents for PC12 cells. This comprises the biological parameters of both survival and neuritogenesis, with the distinction that whereas ATP and other agonists are sufficient to sustain cell viability, they are only subsidiary to promote neuritogenesis. In the presence of sub-optimal NGF concentrations, ATP, ATPγS and 2-Cl-ATP strongly increase neurite outgrowth and, in the absence of NGF, they do promote and sustain durable and robust neurite regeneration, but weakly and transiently induce neurite initiation (D'Ambrosi and Volonté, personal communication; D'Ambrosi et al., 2000). For this reason, they should be considered 'propagators' rather than 'initiators' of neuritogenesis.

As a possible mechanism, we suggest a direct modulation of expression of various P2 receptor subclasses. ATP, like NGF, has been indeed shown here to promote also direct up-regulation of $P2X_2$, $P2X_3$ $P2X_4$, $P2Y_2$ but not $P2Y_4$ subclasses, under neurite-regenerating conditions. While this could depend on increased receptor transcription, as evidenced by semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis during neurite initiation, (data not shown), we could conceive also a combined participation of increased stability or translation of P2 receptors. Moreover, such up-regulation is long lasting (up to 2 days at least), in agreement with the idea of a 'permissive' rather than 'triggering' role for ATP in neuritogenesis. Finally, P2 receptor expression level seems to be correlated to the extent of the neuritogenic response, since ATP further increases neurite regeneration induced by NGF, as well as expression of $P2X_2$, $P2X_3$, $P2X_4$, $P2Y_2$ proteins. Consistently, the neuritogenic-ineffective β,γmet-ATP does not modulate these same receptors. In general, we speculate that such up-regulation might increase the responsiveness to extracellular ATP by augmenting the cellular binding capacity and might therefore be functional to regenerating conditions in the presence of ATP, although a constant receptor expression does not necessary mean lack of involvement.

Considering the co-expression of P2Xr and P2Yr in PC12 cells (Arslan et al., 2000), it seems likely that there is a selective involvement of different P2 receptor subtypes in the diverse steps of neuritogenesis (flattening-adhesion, spiking, progression, stability) and that not only P2X (several subtypes) but also different P2Y receptors might simultaneously be involved. Supportive evidence to this hypothesis is provided by the effectiveness of only ATP, ATPγS and 2-Cl-ATP, which all barely

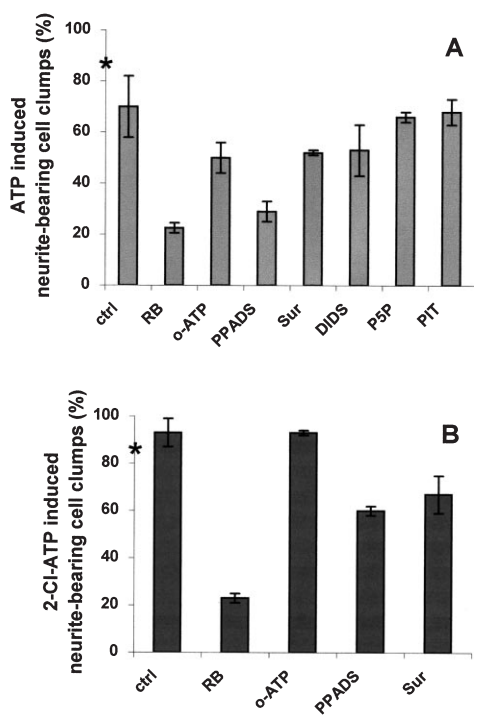

Fig. 4. P2 receptor antagonists prevent ATP and 2-Cl-ATP-induced neurite regeneration. PC12 cells were primed with 50 ng/ml NGF for 1 week, replated for neurite regeneration with 100 μM ATP (A) or 100 μM 2-Cl-ATP (B), in the absence or presence of the following antagonists: (A, B) 10 μM reactive blue 2 (RB), 50 μM *o*-ATP, 30 μM PPADS, 100 μM suramin (Sur); (A) 25 μM 4,4′-diisothiocyanatostilbene-2, 2′disulphonic acid (DIDS), 100 μM pyridoxal-5′-phosphate (P5P) and 10 μM 2,2′-pyridylisatogen (PIT). Neurite-bearing cell clumps were scored after 24 h. Counts represent means ± S.E.M. ($n = 3$). *Stands for NGF value at 24 h. Ctrl, control.

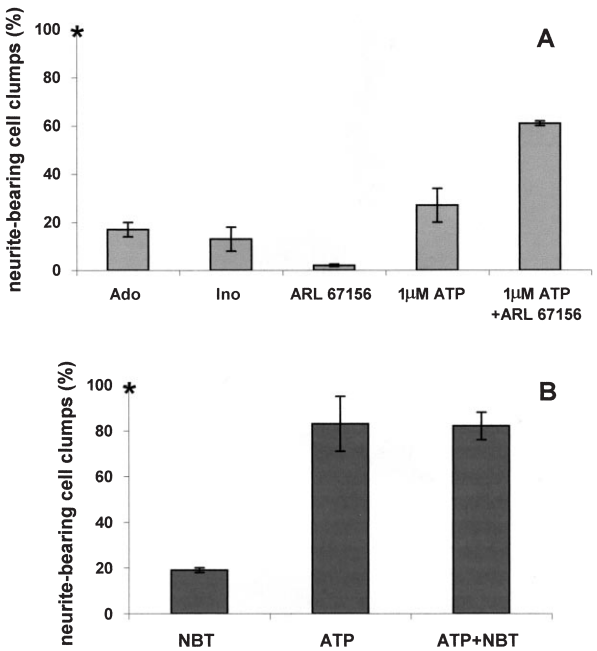

Fig. 5. ATP degradation products are not involved in neurite regeneration. PC12 cells were primed with NGF for 10 days and replated for neurite regeneration with adenosine (Ado), inosine (Ino), ARL 67156 (all at 100 μM), 1 μM ATP and ATP+ARL 67156 (A). (B) Cells were replated with 100 μM ATP, with or without 20 μM NBT. After 24 h, cells were scored for the proportion of neurite-bearing cell clumps. Counts represent means ± S.E.M. ($n = 3$). (A, B) *Represents NGF value, reported as 100% positive control.

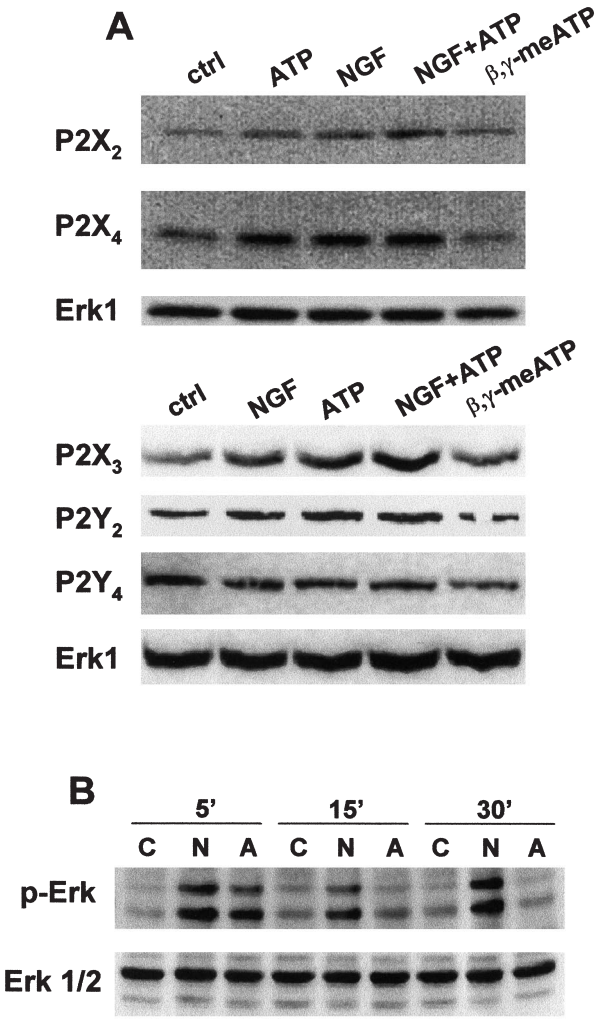

Fig. 6. During neurite regeneration, extracellular ATP up-regulates $P2X_{2,3,4}$, $P2Y_2$ and p-Erk proteins. PC12 cells were primed with NGF for 2 weeks, replated for neurite regeneration with ATP (100 μM), NGF (50 ng/ml) (A, B), β,γmeATP (100 μM) (A). After 24 h ($P2X_3$, $P2Y_{2,4}$) 48 h ($P2X_{2,4}$) (A) or 5, 15 and 30 min (B), cells were lysed and total protein collected in SDS–PAGE sample buffer. Equal proteins were subjected to western blotting and immunoreactions with anti-P2X, P2Y and p-Erk antibodies, all as described in Experimental procedures. Images were analysed using ECL and resolved using Kodak Image Station 440CF. Ctrl/C = Control; N = NGF; A = ATP.

discriminate between P2 receptor subtypes, and whose actions are prevented by different antagonists, not fully overlapping in their inhibitions. Furthermore, by the result that they induce transient (ATP) or long-lasting (2-Cl-ATP) neurite regeneration. However, the identification of the P2 receptor subtypes precisely involved in both progression and maintenance of neuritogenesis still needs to be determined, as do those involved in survival too. In this respect, it should be pointed out that the effects of agonists and antagonists on PC12 cells are the resultant of the simultaneous ligand occupancy of several P2 receptors. Therefore, different P2 receptor combinations would evoke specific different functions.

Parallel modulation of P2 receptor subclasses obtained with NGF and extracellular ATP could imply or a potential direct/indirect cross talk between these receptor systems or a partial overlapping of their down-stream signal transduction pathway(s). The participation of P1 receptors to neurite regeneration, but not to survival (Lee and Chao, 2001; Huang et al., 2001), is instead excluded by the negative results obtained with both adenosine and caffeine. The extracellular action of ATP (but not of its degradation products) is confirmed also by inhibitors of purine nucleosides transport (not blocking ATP-evoked neuritogenesis) and inhibitors of ecto-ATPases (improving the effect of sub-optimal ATP concentrations). Furthermore, whereas recent data established a role for intracellular inosine in neuronal differentiation (Benowitz et al., 1998; Petrausch et al., 2000), this does not appear to be the case for PC12 cells.

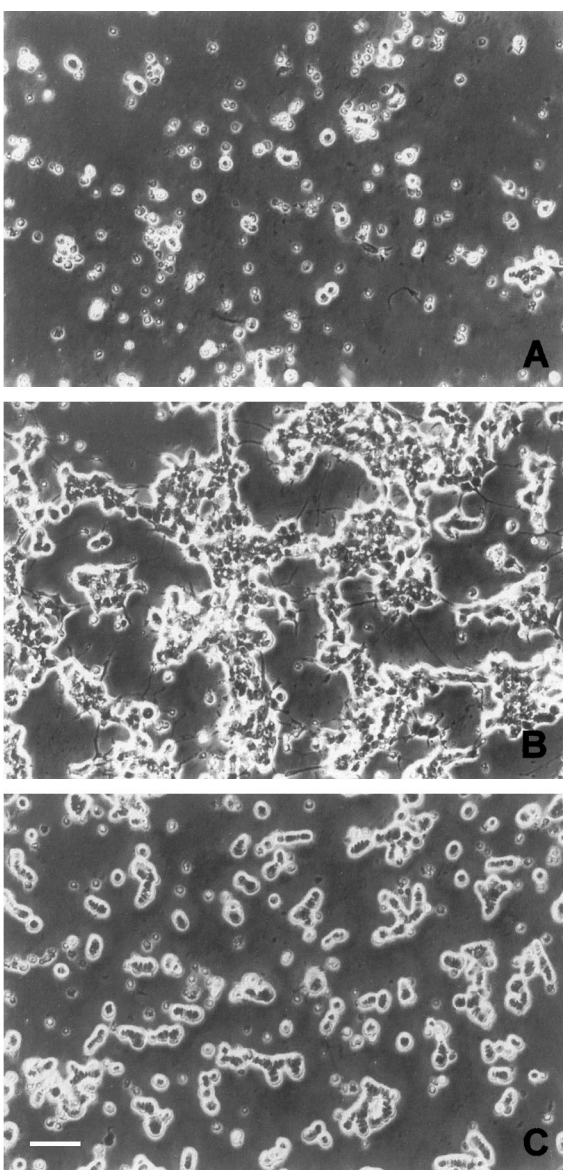

Fig. 7. Extracellular ATP sustains cell survival after serum deprivation. PC12 cells were washed several times by centrifugation–resuspension and replated in serum-free medium, in the presence of NGF 5 ng/ml (A), or 50 ng/ml (B), or 100 μM ATP (C). Photomicrographs were taken after 6 days. Scale bar = 40 μm.

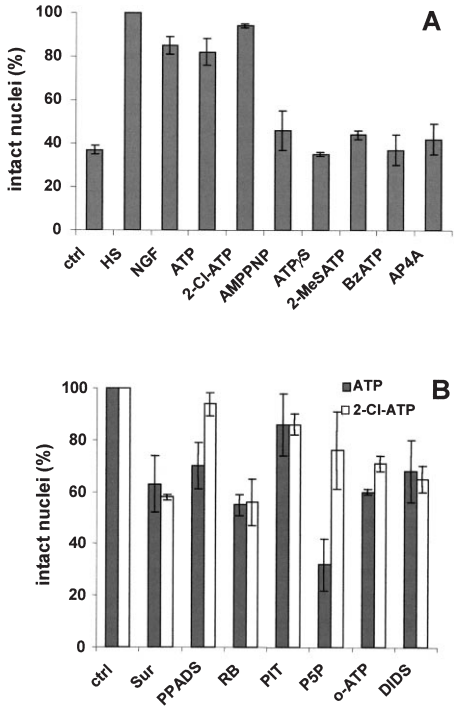

Fig. 8. P2 receptor agonists and antagonists modulate cell survival. PC12 cells were washed several times by centrifugation–resuspension and replated in serum-free medium, in the presence (A, B) of 100 μM ATP, 100 μM 2-Cl-ATP, or (A) 1% horse serum, 50 ng/ml NGF, AMPPNP, ATPγS, 2-MeSATP, BzATP, AP4A, all used at 100 μM, and with the following P2 receptor antagonists (B): 100 μM suramin (Sur), 30 μM PPADS, 20 μM reactive blue 2 (RB), 10 μM PIT, 200 μM P5P, 50 μM *o*-ATP, 10 μM DIDS. Cell survival was assessed 48 h later, by direct count of intact nuclei. Counts represent means ± S.E.M. ($n = 3$) and 100% survival is referred to cultures maintained in 1% horse serum (A) or to cells cultured with ATP or 2-Cl-ATP, respectively (B).

Neuronal cell death mediated by lack of neurotrophic factors is often a consequence of insult or it occurs during normal development and ageing, when there is insufficient supply by the target tissues. It can be accompanied by the presence of extracellular purines released from presynaptic vesicles, from cellular outflow induced by membrane permeability loss, from degradation of nucleic acids of dying cells. We have described here that extracellular ATP, ATPγS and 2-Cl-ATP not only have a role in neuronal repair and regeneration, but they are also permissive to survival of PC12 cells (both naive and NGF-primed) which have been temporarily deprived or insufficiently supplied with growth factors. Therefore, by replenishing a temporary gap in the supply of trophic agents, we suggest that extracellular ATP could come to the rescue and play a key role in survival, repair and remodelling occurring in the nervous system either after injury, or under normal developmental conditions.

In conclusion, we believe that complex biological events such as neuritogenesis and cell survival depend on several different factors. Our data suggest a role for ATP as a trophic agent and the direct involvement of P2 receptors in this function. However, in addition to Erk1-2 tyrosine phosphorylation and activation shown under neurite-regenerating conditions, the proximate targets of P2 receptor modulation in the signal cascade leading to purinergic control of neuritogenesis and survival still need to be determined.

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