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P2 receptor modulation and cytotoxic function in cultured CNS neurons

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Abstract

In this study we investigate the presence, modulation and biological function of P2 receptors and extracellular ATP in cultured cerebellar granule neurons. As we demonstrate by RT-PCR and western blotting, both P2X and P2Y receptor subtypes are expressed and furthermore regulated as a function of neuronal maturation. In early primary cultures, mRNA for most of the P2 receptor subtypes, except $P2X_6$, are found, while in older cultures only $P2X_3$, $P2Y_1$ and $P2Y_6$ mRNA persist. In contrast, P2 receptor proteins are more prominent in mature neurons, with the exception of $P2Y_1$. We also report that extracellular ATP acts as a cell death mediator for fully differentiated and mature granule neurons, for dissociated striatal primary cells and hippocampal organotypic cultures, inducing both apoptotic and necrotic features of degeneration. ATP causes cell death with EC_{50} in the 20-50 μM range within few minutes of exposure and with a time lapse of at most two hours. Additional agonists for P2 receptors induce toxic effects, whereas selected antagonists are protective. Cellular swelling, lactic dehydrogenase release and nuclei fragmentation are among the features of ATP-evoked cell death, which also include direct P2 receptor modulation. Comparably to P2 receptor antagonists previously shown preventing glutamate-toxicity, here we report that competitive and non-competitive NMDA receptor antagonists inhibit the detrimental consequences of extracellular ATP.

Due to the massive extracellular release of purine nucleotides and nucleosides often occurring during a toxic insult, our data indicate that extracellular ATP can now be included among the potential causes of CNS neurodegenerative events. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: P2 receptors; Extracellular ATP; Granule neurons; Hippocampal organotypic cultures; Necrosis; Apoptosis

1. Introduction

In addition to their recognised roles within cells, purine and pyrimidine nucleotides, nucleosides and bases are known to be released in the extracellular space where they act as important intercellular signalling molecules (Vizi and Sperlaugh, 1999; Burnstock and Williams, 2000; Fields and Stevens, 2000; Pintor et al., 2000; Vizi, 2000). Despite the strict regulatory control exerted by ectonucleotidases, which maintain at low levels the physiological concentrations of extracellular purines/pyrimidines (Zimmermann and Braun, 1999), these may reach high levels when released exocytotically from various cell types including neurons, or when flowed out from damaged cells (Agteresch et al., 1999). In the extracellular space they can mediate dual effects: short-term, such as neurotransmission, and long-term, such as trophic action (Chow et al., 1997; Abbracchio and Burnstock, 1998; Rathbone et al., 1999; Burnstock, 1999). Moreover, purines and pyrimidines can interact with other neurotransmitters or growth factors at the signal-transduction level and modify their effects (Neary, 1996; Pankratov et al., 1999). Generally, specific cell-

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Abbreviations: BB, basilen blue; BME, Eagle's basal medium; CGN, cerebellar granule neurons; DIV, days in vitro

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surface purinergic receptors mediate all these functions without requiring ligand metabolism or uptake by target cells (Neary et al., 1996).

Based on pharmacological profiles, on selectivity of coupling to second-messenger pathways and, more recently, on molecular cloning, multiple members within the two main classes of purinergic receptors [P2Y metabotropic, coupled to G-proteins, and P2X ionotropic, ligand-gated ion channels (see Abbracchio and Burnstock, 1994; Ralevic and Burnstock, 1998)], have often been described on the same cell type (Amsallem et al., 1996; Cunha and Ribeiro, 2000; Di Virgilio et al., 2001; Liu et al., 2001; Vassort, 2001). In cultured cerebellar granule neurons (CGN) possessing P1, P2 receptors, as well as ectonucleotidase activities (Dolphin and Prestwich, 1985; Maienschein and Zimmermann, 1996; Kanjhan et al., 1996), we have previously described that the binding of ATP to cell surface receptors displays high and low affinity sites and increases as a function of neuronal maturation (Merlo and Volonté, 1996). The extracellular ATP released from CGN during neuronal differentiation augments the constitutive and KCl/glutamate-evoked D-aspartate outflow (Merlo and Volonté, 1996). Moreover, several P2 receptor antagonists are known to prevent glutamate-evoked Ca^{2+} uptake, neurotransmitter release, excitotoxicity (Volonté and Merlo, 1996) or apoptotic death (serum deprivation-low potassium paradigm, Volonté et al., 1999). They can also rescue CNS, PNS-like neurons from hypoglycaemia and/or chemical hypoxia-evoked cell death (Cavaliere et al., 2001a,b).

In the present study, we characterise the constitutive P2 receptor subtypes present in CGN and address the question whether CGN maturation does influence P2 receptor expression. In addition, we investigate if activation of these receptors by various agonists, in particular the natural ligand ATP, mediates toxicity for CGN or additional primary neuronal targets, such as dissociated striatal primary cells and hippocampal organotypic cultures. Lastly, we approach the dissection of selected mechanistic pathways involved in these actions.

2. Materials and methods

2.1. Dissociated primary cell cultures

CGN from Wistar 8-day-old rat cerebellum were prepared as described (Levi et al., 1989) and seeded on poly-L-lysine-coated dishes, in Eagle's basal medium (BME) (Gibco BRL, MI-Italy), with 25 mM KCl, 2 mM glutamine, 0.1 mg/ml gentamycin, 10% heat inactivated foetal calf serum (Gibco BRL, MI-Italy). At 1 DIV, cultures were supplemented with 10μM cytosine arabinoside and kept for 9 days, without replacing the culture medium.

2.2. Organotypic cell cultures

Organotypic hippocampal slice cultures were prepared using a modification of the Stoppini method (Stoppini et al., 1991). Briefly, hippocampi were dissected out from Wistar rat pups (8–10 days old), cut with Mc Ilwain tissue chopper (400 μm) and separated with cold HBSS (0.185 mg/ml $CaCl_2$, 0.1 mg/ml $MgSO_4$, 0.4 mg/ml KCl, 0.06 mg/ml KH_2PO_4, 8 mg/ml NaCl, 0.05 mg/ml Na_2HPO_4, 0.35 mg/ml $NaHCO_3$, 1 mg/ml glucose). Four slices were plated on each Millicell CM culture inserts (Millipore, RM-Italy) and maintained in organotypic culture medium (50% BME, 25% HBSS, 25% heat inactivated horse serum, supplemented with 4.5 mg/ml glucose, 1 mM glutamine) at 37°C and 5% CO_2. Medium was changed every 3–4 days and experiments performed after 14 DIV. ATP (2mM) was added to the medium, in the presence of 6 μg/ml propidium iodide; neuronal damage was assessed 20 h later with a fluorescence microscope.

2.3. Total RNA isolation

CGN were lysed in TRI REAGENT® (Molecular Research Center, Inc.) and total RNA was extracted following the manufacturer's instructions. After isolation, RNA samples were digested for 30 min at 37°C with 2 units of DNase (RQ1-Promega, Mi-Italy) in DNase buffer (40 mM Tris-HCl, pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$, 10 mM $CaCl_2$), in the presence of 10 mM DTT and 100 units of ribonuclease inhibitor (RNAguard-Pharmacia Biotech).

2.4. Reverse transcription and PCR

Total RNA isolated from CGN was subjected to RT for 50 min at 42°C using SuperScript™ II (200 units) and First Strand Buffer (Life Technologies, Mi-Italy), in the presence of 25 mM oligo dT, 10 mM DTT and 500 nM dNTP mix. Amplifications of cDNA obtained from RT were then performed in PCR buffer (Amersham Pharmacia Biotech, Mi-Italy) containing dATP, dGTP, dTTP, dCTP (each at 400 nM), in the presence of 1 μM of each specific primer and 2.5 units of Taq DNA Polymerase (Amersham Pharmacia Biotech). PCR products were obtained after 30 cycles, at annealing temperatures ranging from 54 to 60°C (Table 1). Nested PCR were performed to amplify P2Y mRNAs, using in a first step external degenerated primer mix (annealing at 45°C to sequences common to all P2Yr) (sense: 5′-(A/G)T(C/G/T)(C/T)T(A/C/G/T)TT(C/T)CT(C/G)ACCT G(C/T)AT-3′; antisense: 5′-(A/C/G)A(C/G) (A/C/G/T)A(C/T)(A/G/T)GG(A/G)TC(A/C/G)A(A/C/G)

Table 1
P2Xr and P2Yr primers: sequences, annealing temperatures, sources and product lengths

P2X/Y PRIMERS	Tm (°C)	Source	cDNA (bp)
X1S 5′ TGGATGACAAGATCCCAAGC 3′ **X1A** 5′ ACGGTTTGTCCCATTCTGCA 3′	60	Norway rat vas deferens	418
X2S 5′ ATGGAACTTCTGACAACCAT 3′ **X2A** 5′ AGTGGTGGTAGTGCCGTTTA 3′	60	Norway rat cerebellum	418
X3S 5′ CAACTTCAGGTTTGCCAAA 3′ **X3A**5′ TGAACAGTGAGGGCCTAGAT 3′	55	Arslan et al., 2000	519
X4S 5′ ACGACGTTGGCGTGCCAACG 3′ **X4A** 5′ GCGCTGCTCTTTGCCGGCCA 3′	60	Norway rat brain	411
X5S 5′ CAAAGTCCATGCCAACGGAT 3′ **X5A** 5′ ACGGAACTCTACCCCATTAG 3′	55	Norway rat heart	421
X6S 5′ GTAGTGCTGTGCCCAGGAAA 3′ **X6A** 5′ GGACTCCACGCCTGAGGCTG 3′	60	Norway rat brain	418
X7S 5′ AAGGGAAAGAAGCCCCACGG 3′ **X7A** 5′ CCGCTTTTCCATGCCATTTT 3′	60	Norway rat macrophage	418
X8S 5’ CATGCCATGGGGCAGGTGTCCTGGAAG 3’ **X8A** 5’ GGCTTGGATCTTTTCTCCAC 3’	60	Bo et al., 2000	529
Y1S∗ 5′CAAGTCTCTGGGCAGGCTCA 3′ **Y1A**∗ 5′ AGGTGGCATAAACCCTGTCG 3′	64	Norway rat insulinoma cells	451
Y1S° 5′CATCTCCCCCATTCTCTT 3′ **Y1A**° 5′ GTTGCTTCTTCTTGACCTGT3′	54	Arslan et al., 2000	663
Y2S∗ 5′ TCTGCACTCCCTGAGCTGGG 3′ **Y2A**∗ 5′ ACGCCATGTTGATGGCGTTG 3′	64	Norway rat pituitary gland	442
Y2S° 5′ ACCCGCACCCTCTATTACT3′ **Y2A**° 5′ CTTAGATACGATTCCCCAACT3′	56	Arslan et al., 2000	538
Y3S∗ 5′ GTGGCACAAAAAGAAGGGAA 3′ **Y3A**∗ 5′ AGGCAATGGCAAAAGCCTGC 3′	60	Chicken brain	445
Y4S∗ 5′ GCCCTCGATTTGCAAGCCTT 3′ **Y4A**∗ 5′ AAACCACATTGACAATGTTC 3′	55	Norway rat heart	428
Y6S∗ 5′ CTGGCACAAGCGTGGAGGTC 3′ **Y6A**∗ 5′ AGGCTGCAGCGAAGGTCTCC 3′	64	Norway rat aorta	445
Y6S° 5′GTTATGGAGCGGGACAATGG 3′ **Y6A**° 5′ AGGATGCTGCCGTGTAGGTT3′	59	Arslan et al., 2000	347

∗ indicates primers used in nested PCR; ° indicates primers used in semi-quantitative PCR.

(A/G)(A/C)(A/C)(A/G)C(A/T)(A/G)TT-3′); a second step was obtained with the previous PCR product as template and with the specific internal P2Y primers (marked with the symbol ∗ in Table 1). To test genomic DNA contamination, aliquots of each total RNA were processed in the absence of the reverse-transcriptase enzyme, whereas each PCR was performed in the absence of template, to avoid cross-contamination. Amplification products and 50/100 base-pair marker (1μg) were electrophoresed on 1% agarose gel containing ethidium bromide (1 μg/ml) and photographed under UV light, using Kodak Image Station 440. PCR experiments were performed with at least three different CGN preparations.

2.5. Semi-quantitative PCR

Equal amount of total RNA (1 μg) extracted from CGN at 2–9 DIV was subjected to RT and 50 ng of each cDNA were amplified with P2 receptor specific primers (P2Y oligonucleotides used are marked with the symbol ° in Table 1). The number of cycles for each PCR, varying from 25 to 30, was fixed in a range of linear activity of the DNA polymerase, in order to ensure a proportional ratio between the amount of specific mRNA and the number of DNA molecules obtained from the amplification. Moreover, to asses that same quantity of DNA was amplified in each reaction, cyclofilin primers (sense: 5′-TTGTCCATGGCAAATGCTGGACCA-3′; antisense: 5′-TGCTCTCCTGAGCTACAGAAGGAA-3′) were inserted in each PCR for 18 cycles. GAPDH (Clontech Labs. Inc., Palo Alto-CA) and β-actin primers (sense: 5′-ATGGATGACGATATCGCTGC-3′; antisense: 5′-TCATAGATGGGCACAGTGTG-3′) were also tested. PCR products (15 μl out of 50) were run on 5% acrylamide gel (acrylamide:bis-acrylamide, 29:1), stained with ethidium bromide (1 μg/ml), acquired under UV light and quantified by Kodak Image Station 440. Moreover, after fixing (95% EtOH, 5% acetic acid, 10 min), the same gels were silver stained with 0.1% $AgNO_3$ (15 min) and developed in 0.25 M NaOH,

0.0025% formaldeyde. Bands were quantified by Kodak Image Station 1440.

2.6. Survival studies

CGN were incubated for different times with extracellular ATP or other analogs, as described, in Earle's Balanced Salt Solution (116 mM NaCl, 25 mM KCl, 1.8 mM CaCl2, 0.8 mM MgSO4, 26 mM NaHCO3, 0.6 mM NaH2PO4, pH 7.4), supplemented with 1 mg/ml glucose. Additional agents were added under different conditions, as indicated. The cells were then returned to the previously saved culture medium and survival evaluated by direct count of intact nuclei (Volonté et al., 1994, Atlante et al., 2000; Stefanis et al., 1998). Briefly, the procedure is based on the solubilization of cellular membranes by a mild detergent, followed by direct count of all intact nuclei. When originating from healthy cells, these last are in fact fully preserved despite the use of the detergent.

2.7. Chromatin staining by Höechst 33258

CGN at 9 DIV were exposed to 500 μM extracellular ATP for different times, and shifted to the previously saved conditioning medium for 2 h. They were fixed for 20 min in 4% paraformaldehyde, washed three times with PBS, permeabilised in 0.2% Triton. Chromatin was visualised by fluorescence microscopy using 1μg/ml Höechst 33258 (Ex. at 345, Em. at 478 nm).

2.8. SDS-PAGE, western-blotting

Protein concentration was determined by the Bradford method (Bradford, 1976). Analysis of protein components was performed on 12% polyacrylamide gels, as described (Laemmli, 1970), loading the same amount of total protein for each experimental condition. Proteins were transferred to PVDF membranes (Amersham) and blots were probed for 2 h at room temperature with the specified antisera, followed by horseradish peroxidase-coupled secondary antibody, and analysed using ECL chemiluminescence (Santa Cruz, Mi-Italy). Quantification was performed by Kodak Image Station (KDS IS440CF 1.1). $P2X_{1,2,4,7}$ receptor antisera (from Alomone, Jerusalem, Israel) were used at 1:200 dilution. $P2Y_1$ was used at 1:400, $P2Y_4$ at 1:300 and $P2X_3$ at 1:2000 dilution. As specified in the certificate of analysis provided for each antisera, all antibodies were affinity purified and raised against highly purified peptides (identity confirmed by mass-spectrography and amino acid analysis), corresponding to specific epitopes not present in any other known protein ($P2X_1$:382-399; $P2X_2$:457-472; $P2X_3$:383-397; $P2X_4$:370-388; $P2X_7$:576-595; $P2Y_1$:242-258; $P2Y_4$:337-350). Specificity of each P2r signal was also directly tested by immunoreactions in the presence of neutralising peptides (ratio 1:1 between peptide and antiserum). The secondary antisera were used at 1:15000 dilution.

2.9. Statistical analysis

Statistical differences were verified by one-way analysis of variance and $p<0.05$ was considered as statistically significant: $*$ $p<0.05$, $**$ $p<0.01$, $***$ $p<0.001$.

3. Results

3.1. Presence and modulation of P2X and P2Y receptors in cerebellar granule cultures

We have screened CGN at maturation (8–9 DIV) for the presence of P2 receptors, using RT-PCR with primers specific for various subclasses of P2X and P2Y (Table 1). Bands corresponding to mRNA for $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_7$, but not $P2X_6$ are found as well as mRNAs for $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ (Fig. 1). These primers do not generate additional bands and the sequenced PCR products provided an average sequence-homology to cloned P2 receptor always higher than 90% (data not shown).

As measured by semi-quantitative RT-PCR analysis using cyclofilin as internal standard, CGN maturation and differentiation affects the content of several P2 receptor mRNAs (Fig. 2). Thus, in mature granule cultures (8–9 DIV) a significant decrease is found for $P2X_1$, $P2X_4$, $P2X_5$, $P2X_7$, $P2Y_2$ and $P2Y_6$ mRNAs (about 80, 65, 80, 75, 84 and 50% respective inhibition, at 8-9 compared to 2 DIV). Whereas $P2X_3$ and $P2Y_1$ mRNAs remain approximately constant during development, $P2X_2$ is instead the only that transiently increases (up to 3-fold at 5 DIV, compared to 2 or 8 DIV, Fig. 2). We have also examined the expression of two unrelated

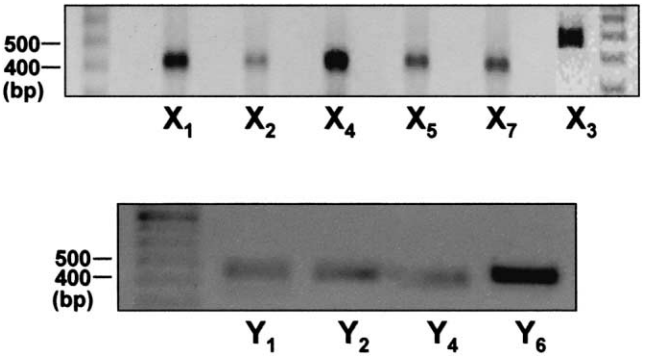


Fig. 1. P2 receptor mRNAs are expressed in cerebellar granule cultures: overview by RT-PCR analysis. Total RNA isolated from 8 DIV-CGN was subjected to RT and the resulting DNA was amplified using primers specific for each P2 receptor (see Table 1). PCR products were resolved on 1% agarose gel and visualised by ethidium bromide staining.

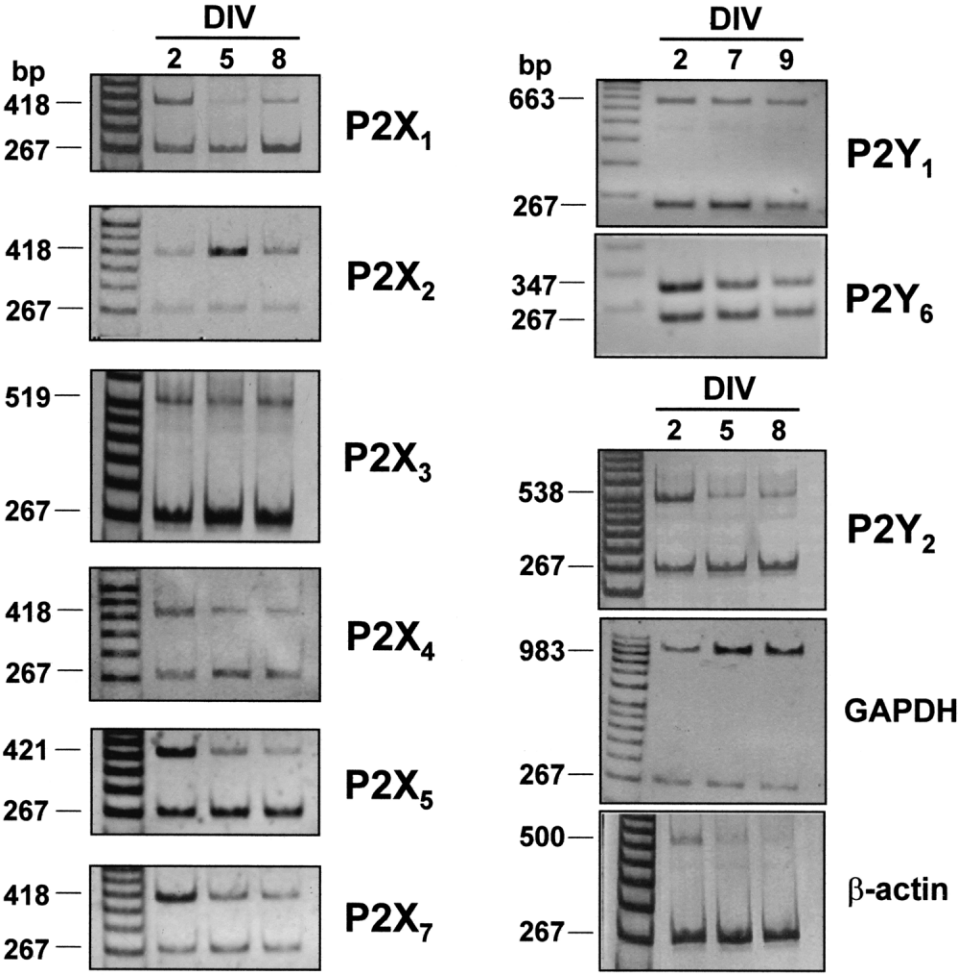


Fig. 2. Differential expression of P2X and P2Y mRNAs as a function of neuronal maturation. DNA obtained from equal amounts of total RNA extracted from CGN at 0-9 DIV was semi-quantitatively amplified in the presence of cyclofilin as internal standard and run on 5% polyacrylamide gel. Samples were visualised by ethidium bromide staining ($P2X_{2/4/5/7}$, $P2Y_{1/6}$, GAPDH), followed by silver staining ($P2X_{1/3}$, $P2Y_2$, β-actin). Similar results were obtained in two independent experiments.

genes and we find that whereas β-actin diminishes as a function of neuronal maturation (about 55% between 2 and 8 DIV), GAPDH augments (about 3-fold between 2 and 8 DIV).

These results are completed by P2 receptor protein analysis obtained by western blotting (Fig. 3). Quite differently from the corresponding mRNAs, we show that the protein content of all P2 receptor analysed drastically increases, with the only exception of $P2Y_1$. In detail, $P2X_1$ protein is almost undetected at 0–2 DIV but 3-fold increased between 4 and 9 DIV. $P2X_{2,3}$ are hardly detected at 0 DIV, but progressively augmented thereafter (about 8,16-fold between 0 and 9 DIV). $P2X_4$ is already detected at 0 DIV, with a 5-fold progressive increase at 9 compared to 2 DIV. $P2X_7$ is induced about 50-fold (between 2 and 9 DIV) and $P2Y_4$ protein shows about 18-fold increase between 2 and 9 DIV. At last, $P2Y_1$ protein is about 10-fold decreased between 0-2 and 9 DIV. We have positively established the specificity of each signal, by performing immunoreactions in the presence of neutralising peptides for each P2 receptor subtype (Fig. 3, lane 9+). Moreover, the molecular masses of all P2 receptors detected by the antisera are in agreement with results from other cell types and cloning data (Brake et al., 1994; Valera et al., 1994; Bo et al., 1995; Surprenant et al., 1996; Collo et al., 1997; Vulchanova et al., 1997; Chan et al., 1998; Bogdanov et al., 1998; Lê et al., 1998; Webb et al., 1998). In Table 2, a synoptic view of P2 receptor expression/modulation is provided. We have then compared these results with the tyrosine/threonine phosphorylation of signal transduction markers involved in development and differentiation, ERK1/2 kinases (Cavanaugh et al., 2001). We find that ERK1/2 are also activated as a function of neuronal maturation (respectively 8–13-fold induction between 2 and 9 DIV) (Fig. 3).

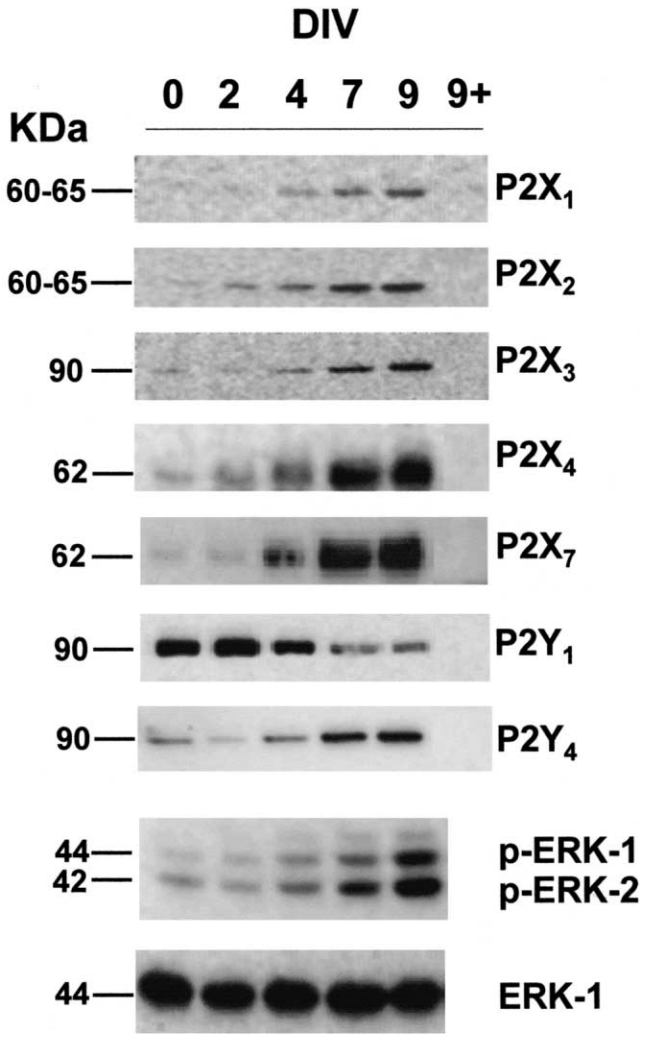


Fig. 3. Differential expression of P2X and P2Y proteins as a function of neuronal maturation. Total protein from CGN at 0-9 DIV was extracted, subjected to SDS-PAGE, Western blotting, immunoreactions and analysed using ECL chemiluminescence, all as described in the Methods section. Similar results were obtained in three independent experiments. The lane 9+ represents immunoreactions from CGN at 9 DIV, performed in the presence of neutralising peptides for each P2 receptor.

3.2. Detrimental function of extracellular ATP in cerebellar granule, striatal dissociated and hippocampal organotypic cultures

As determined by direct count of intact nuclei up to 20 h post-treatment, exposure of mature CGN to extracellular ATP leads to cell death affecting 65–95% of the entire neuronal population. Time-course experiments indicate that cell death reaches ~50% in two minutes of treatment and is maximal in approximately 5 min (Fig. 4A). Moreover, complete cell death occurs within a time lapse of at most 2–3 h post-treatment (Fig. 4B). Examining the dependence of this event upon neuronal maturation, in accordance with P2 receptor protein expression (Fig. 3), we observe that CGN are resistant to ATP during the first week in culture (Fig. 4C). Cell death is then maximally obtained at 100 μM ATP, with EC_{50} in the 20–50 μM range (Fig. 5A). Different P2 receptor agonists were tested (Fig. 5B), the most detrimental appearing adenosine 5′-O-(3-thiotriphosphate) (about 95% cell death, at 500 μM), 2-Cl-ATP (~80%, at 500 μM) and 3′-O-(4-benzoyl) benzoyl ATP (~50%, up to 500 μM). The agonists 8-Br-ATP and 2-methylthio-ATP elicit only mild actions (35–10% cell death at 500 μM), whereas adenosine (not shown), AMP, ADP, 5′-adenylyl-β,γ-imidodiphosphate (AMPPNP), adenosine-5′-tetraphosphate, α,β-methylene-ATP, β,γ-methylene-ATP and UTP (all used up to 500 μM) are ineffective. Although the magnitude and time courses of cell death depend on each single effective compound, the rank order potency observed with the different nucleotides is similar for all cell preparations. Among the antagonists tested, we find that the sulfonic derivative of anthraquinone basilen blue (BB) (Burnstock and Warland, 1987) and suramin (Hoyle et al., 1990), both of which do not fully discriminate between P2X and P2Y receptors, elicit respectively 90–100% and ~75% neuroprotection (Fig. 5C). The antagonists pyridoxal-5-phosphate (P5P) (Trezise et al., 1994), pyridoxal-phosphate-6-azophenyl-2′,4′-disulphonic acid 4-sodium (PPADS) (Ziganshin et al., 1994), 4,4′-diisothiocyanatostilbene-2, 2′disulphonic acid (DIDS) (Soltoff et al., 1998) and calmidazolium (Virginio et al., 1997) consistently protect CGN from cell death by ~80%, 35%, 30% and 20%, respectively. Caffeine (adenosine receptor antagonist, not shown), ATP-2′,3′-dialdehyde (irreversible $P2X_7$ receptor inhibitor, Murgia et al., 1992) and KN62 (1-(N,O-bis[5-Isoquinolinesulphonyl]-N-methyl-L-tyrosyl)-4phenylpiperazine) (Humphreys et al., 1998) fail under this regard (Fig. 5C).

We have then investigated the detrimental actions of ATP on additional dissociated primary neuronal cultures from the striatum, also known to possess P2 receptors (Sorimachi et al., 2000). After a 15 min exposure to 500 μM ATP, approximately half of the population is found susceptible to cell death (data not shown). Organotypic primary cultures from hippocampus are also sensitive to ATP-evoked toxicity (2 mM, 18 h) (Fig. 6). As detected by propidium iodide incorporation, we observe neuronal damage especially in the dentate gyrus and CA3 areas (Fig. 6). This effect is drastically reduced by the simultaneous presence of BB.

3.3. Features and mechanisms of ATP-evoked cell death

The addition of lethal concentrations of ATP induces in CGN visible necrotic as well as apoptotic morphological changes. Within a few minutes of ATP treatment, most of the cells already show distinctly swollen volumes, progressive darkened cell bodies, formation of blebs on the plasma membrane and loss of phase-bright-

Table 2
Synoptic view of P2 receptor expression and modulation in CGN

P2r	0 DIV	2 DIV	4/5 DIV	7 DIV	8/9 DIV	Trend
X1-m		+++	+		+	↓
X1-p	−	−/+	+	++	+++	↑
X2-m		+	+++		++	↗
X2-p	−/+	+	++	+++	+++	↑
X3-m		++	++		++	→
X3-p	−/+	−/+	+	++	+++	↑
X4-m		++	+		+	↓
X4-p	−/+	+	++	+++	+++	↑
X5-m		+++	+		+	↓
X7-m		+++	+		+	↓
X7-p	−/+	−/+	++	+++	+++	↑
Y1-m		++		++	++	→
Y1-p	+++	+++	++	+	+	↓
Y2-m		+++	+		+	↓
Y4-p	+	−/+	+	+++	+++	↑
Y6-m		+++		++	+	↓

Net intensity of each mRNA (m) or protein (p) band was quantified and proportionally expressed with the + symbols relative to each P2 receptor subtype. The symbol → represents a constant trend during CGN development; the symbols ↓,↑ stand for constant decrease or increase, respectively; ↗ stands for transient increase.

ness (Fig. 7, panels a,c). Nuclear chromatin instead aggregates into a *morula*-like mass with no discernible fine structure and afterwards we observe nuclei condensation and fragmentation (Fig. 7, panels b,d). The surface blebs eventually separate forming apoptotic bodies and fully fragmented nuclei (see arrows). Besides producing the features of necrosis and apoptosis described above, exposure of CGN to extracellular ATP causes a simultaneous extracellular release of the cytosolic enzyme lactic dehydrogenase, which is about 50% of that induced by high glutamate and is totally prevented by BB (not shown).

3.4. Toxic extracellular ATP modulates P2X and P2Y proteins

We know that hypoglycaemia induces CGN cell death also increasing $P2X_{4,7}$ protein expression (Cavaliere and Volonté, personal communication). As detected by western blotting (Fig. 8), we now show that $P2X_1$, $P2X_4$, $P2Y_1$ and $P2Y_4$ proteins are slightly up regulated by toxic extracellular ATP (about 30%, 20%, 15% and 90% increase, respectively), whereas $P2X_{2,3}$ are barely inhibited (27–35%). $P2X_7$ is instead the most significantly affected, eliciting up to 6-fold increase in 30 min (Fig. 8). Nevertheless, $P2X_7$ increased protein expression does not depend on augmented mRNA transcription, as shown from semiquantitative RT-PCR analysis (Fig. 2), and we do not exclude increased translation and/or stability of the protein. Moreover, the addition of lethal concentrations of ATP induces in CGN the intracellular tyrosine/threonine phosphorylation and activation (up to 2–3-fold) of both ERK1/2 kinases (Fig. 8), as shown during neuronal maturation (Fig. 3).

3.5. Role of glutamate receptors

Since different P2 receptor antagonists prevent ATP- (Fig. 5C) and glutamate-evoked cell death (Volonté and Merlo, 1996; Volonté et al., 1999), we have also tested the effect of glutamate antagonists on detrimental actions induced by ATP (Fig. 9). We find that the competitive NMDA receptor antagonists 2-amino-5-phosphonopentanoic acid (AP5) and 3-(2-carboxypiperazin-4-yl) propyl-1-phosphonic acid (CPP) at 10 μM sustain respectively ~50 and 90% neuroprotection. Also effective is the non-competitive antagonist hydrogen maleate (MK801) (85-90% protection) and the antagonist 3-amino-1-hydroxy-2-pyrrolidone (HA966), acting at the glycine site of glutamate receptor (40% neuronal survival), when used up to 10 μM. Finally, the inhibitor of neurotransmitter release riluzole (50 μM) prevents neuronal damage and cell death in ~40% of CGN (Fig. 9).

4. Discussion

The characterisation of ATP receptors mediating specific biological responses in target cells has been complicated by the existence of only a few selective antagonists and by the co-expression of multiple subtypes in the same tissues and cells (Ralevic and Burnstock, 1998; Jacobson et al., 1999). Using different approaches, mainly RT-PCR and western blotting, we have described

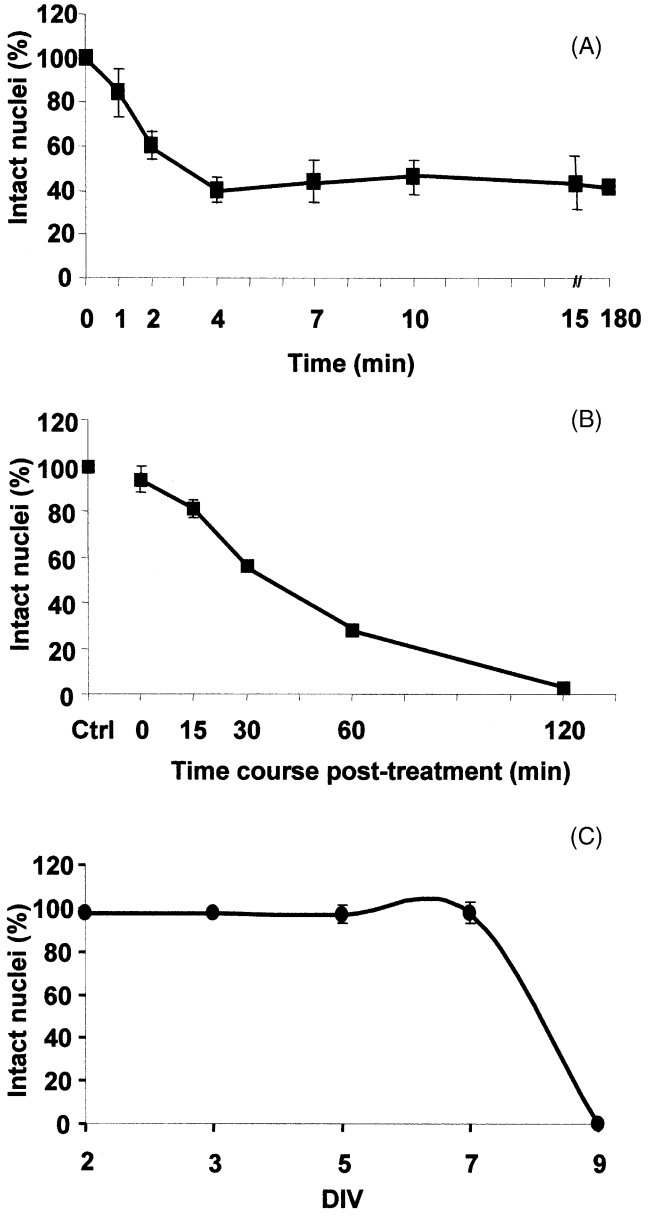


Fig. 4. Extracellular ATP is toxic for cerebellar granule cultures: time course effects. In (A), CGN at 9 DIV were exposed to 300 μM ATP for different times and returned to their conditioned medium for 18 h. Cell survival was evaluated by direct count of viable intact nuclei. In (B), CGN were exposed to 500 μM ATP for 15 min and cell survival was evaluated at different times post-treatment. In (C), CGN at different days in culture were exposed to 300 μM ATP for 15 min and cell survival was evaluated two hours after treatment. All data represent means ± SEM (n=6) and statistical differences were verified by one-way analysis of variance. Each experimental point provided $p<0.001$, when compared with Control (100%) value.

in this paper that also CGN, the major neuronal phenotype of the CNS, express multiple P2 receptor subtypes, belonging to both P2X and P2Y gene families. In detail, $P2X_1$, $P2X_2$, $P2X_3$, $P2X_4$, $P2X_5$, $P2X_7$ subclasses, as well as $P2Y_1$, $P2Y_2$, $P2Y_4$ and $P2Y_6$ are found and differently modulated during neuronal development. Expression patterns for these receptors show that transient or sus-

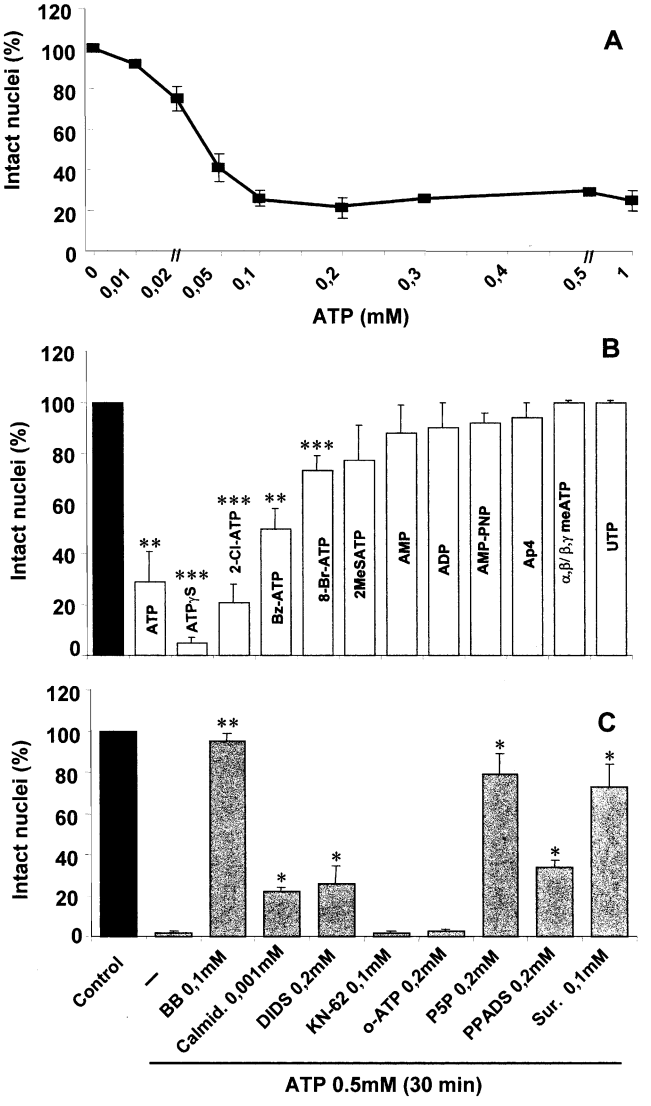


Fig. 5. P2 receptor agonists and antagonists can modulate cerebellar granule culture survival. In (A), CGN at 9 DIV were exposed to different concentrations of extracellular ATP for 7 min. Cell survival was evaluated 2 h after treatment. In (B), CGN were exposed to different P2 receptor agonists for 30 min and cell survival was evaluated 2 h after treatment. In (C), CGN were exposed to 500 μM ATP for 30 min, in the simultaneous presence of various P2 receptor antagonists. Cell survival was evaluated 2 h after treatment. Data represent means ± SEM (n=6). In (A), each experimental point provided $p<0.001$ by one-way analysis of variance, when compared with Control (100%) value. In (B), statistical differences were verified versus the Control (100%) value and in (C), versus the ATP value. ATPγS: adenosine 5'-O-(3-thiotriphosphate); Bz-ATP: 3'-O-(4-benzoyl) benzoyl ATP; 2-MeSATP: 2-methylthio-ATP; AMP-PNP: 5'-adenylyl-β,γimidodiphosphate; Ap4: adenosine-5'-tetraphosphate; α,βmet-ATP: α,β-methylene-ATP; β,γmet-ATP: β,γ-methylene-ATP; Calmid: calmidazolium; DIDS: 4,4'-diisothiocyanatostilbene-2, 2'disulphonic acid; KN-62: 1-(N,O-bis[5-Isoquinolinesulphonyl]-N-methyl-L-tyrosyl)-4-phenylpiperazine; o-ATP: ATP-2′,3′-dialdehyde; P5P: pyridoxal-5-phosphate; PPADS: pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid 4-sodium; Sur: suramin.

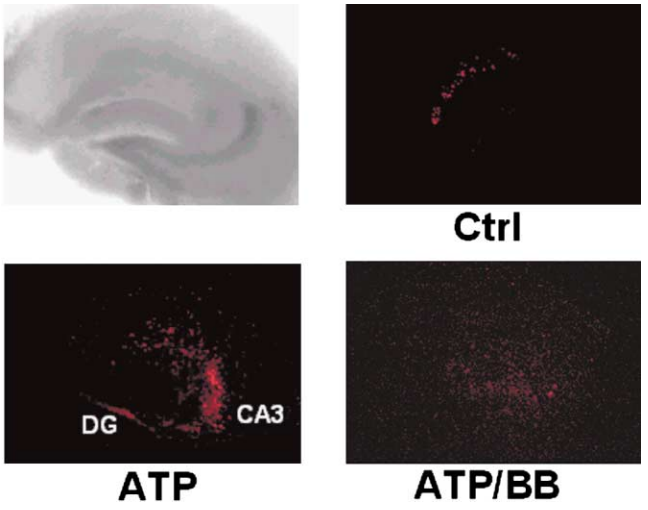


Fig. 6. Extracellular ATP induces cell death in organotypic hippocampal cultures. Organotypic hippocampal cultures at 14 DIV were exposed to 2 mM extracellular ATP for 18 h, with or without 100 μM BB and in the simultaneous presence of 6 μg/ml propidium iodide. Cellular damage was visualised using Zeiss fluorescence microscope. Similar results were obtained in two independent experiments.

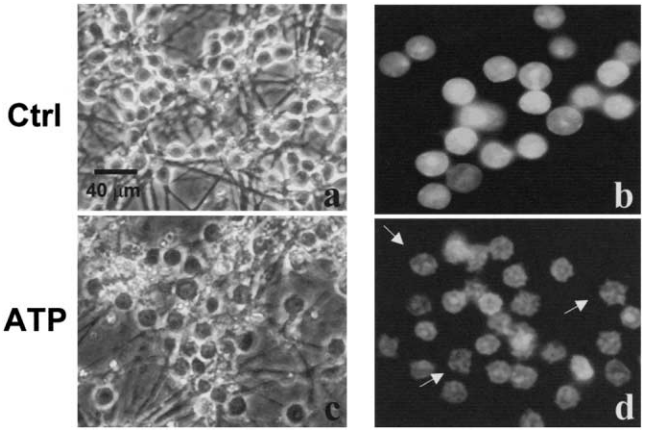
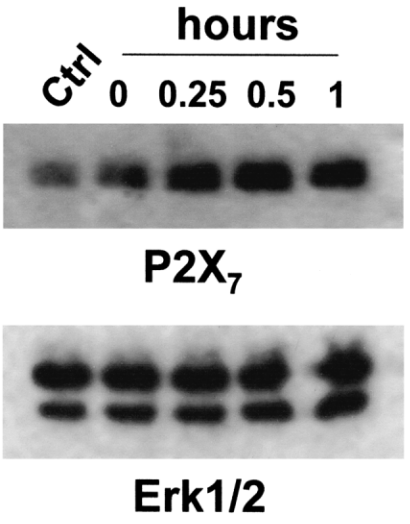


Fig. 7. Extracellular ATP provokes cellular swelling and nuclei condensation-fragmentation. CGN at 9 DIV were exposed to 500 μM ATP for 30 min (panels c,d). In (a,c), photomicrographs were taken 15 min post-treatment using Nikon SC35 camera (scale bar = 40 μm). In (b,d), chromatin was visualised by staining with Höechst 33258 (1 μg/ml) and using Zeiss fluorescence microscope. Similar results were obtained in three independent experiments.

tained regulation of different P2 subtypes, as well as absence of modulation, can occur simultaneously. Generally, P2 receptor mRNAs are expressed strongly during earlier stages of differentiation, with the exceptions of $P2Y_1$ and $P2X_3$ mRNAs, which are more invariably expressed throughout development, and $P2X_2$ mRNA, which is the only transiently increased during maturation. This reflects a pattern which is not unusual for neuronal development and is not due to progressive growth decline in culture conditions (Przyborski and Cambray-Deakin, 1995; Uberti et al., 1998). P2 receptor proteins appear instead to be more prominently represented at complete maturation, with the exception of $P2Y_1$. The reason for these trends is not clear, but we speculate that a mechanism of reciprocal switching on/off may occur, being the receptors sequentially turned on to probably generate a well-defined P2 receptor expression wave. A similar progression is already established also for glutamate receptors, in which mGluR5 increases with neuronal maturation of CGN, whereas mGluR1α is instead always constant (Masgrau et al., 2001).

In parallel with P2 receptor maximal protein expression during late development, we also observed here that detrimental actions exerted by extracellular ATP only occur on mature CGN. These events are concurrent with NMDA receptor modulation (Burgoyne et al., 1993) and sensitivity to the cytotoxic glutamate action (Balàzs et al., 1992). In several neuronal populations excitotoxicity largely mediated by ionotropic glutamate receptors in turn modulates purine release (Inoue et al., 1995; Jin and Fredholm, 1997; Robertson and Edwards, 1998; Bennett and Boarder, 2000). On the other hand, we have previously shown that extracellular ATP promotes glutamate release in CGN (Merlo and Volonté, 1996) and, in addition, neuronal maturation and differentiation per se augment ATP release and binding to CGN (Merlo and Volonté, 1996). Taken together this would suggest that excitatory amino acids and purines can be considered as separate components of a single functional unit that, under physiological and/or pathological conditions, can regulate the efficiency of the synaptic transmission. Thus, excitatory amino acids and purines would act in synergism (Inoue et al., 1992, 1995; Fujii et al., 1999; Pankratov et al., 1999) to guarantee and reinforce a biological function that, in the specific case of CGN, we have shown here to correspond to evoked cell death. This hypothesis is reinforced by the experimental evidence that, whereas P2 receptor antagonists do constrain CGN cell death induced by high glutamate (Volonté and Merlo, 1996; Volonté et al., 1999), NMDA antagonists have the same action against ATP-dependent cell death. Therefore, the release of ATP from mature neurons could very likely participate, together with glutamate, to neuronal degeneration following acute and chronic insults. As a matter of fact, ATP is known to be released in vivo in the extracellular environment of the CNS, under both physiological and pathological conditions (Lutz and Kabler, 1997; Phillis et al., 1993). A similar interplay between purines and growth factors has already been described to promote, for example, mitogenic actions in fibroblasts (Wang et al., 1994), gliotic responses in astrocytes (Bolego et al., 1997) or neuritogenesis in sympathetic-like PC12 cells (D'Ambrosi et al., 2000, 2001).

Thus, several biological responses typically mediated by high glutamate such as cellular swelling, lactic dehydrogenase release, ERKs activation (Michaelis,



	Ctrl ATP	% variation
$P2X_1$		30
$P2X_2$		-27
$P2X_3$		-35
$P2X_4$		20
$P2Y_1$		15
$P2Y_4$		87
p-ERK-1 p-ERK-2		250 200

Fig. 8. P2 receptor protein modulation by extracellular ATP. CGN at 9 DIV were maintained for 15 min ($P2X_7$) or 30 min ($P2X_{1/2/3/4}$, $P2Y_{1,4}$, p-ERK-1/2) in the presence of 500 μM extracellular ATP. Total protein was extracted at different time post-treatment ($P2X_7$) or immediately after treatment ($P2X_{1/2/3/4}$, $P2Y_{1,4}$, p-ERK-1/2) and 30 μg subjected to SDS-PAGE and western-blotting with the specified antisera. Quantitative analysis was performed after normalisation, by Kodak Image Station (KDS IS440CF 1.1). Similar results were obtained in two independent experiments.

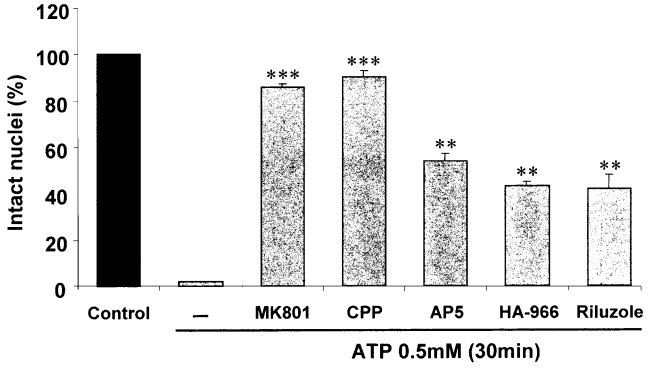


Fig. 9. Glutamate antagonists prevent ATP-evoked cell death. After 30 min pre-treatment with glutamate antagonists, CGN at 9 DIV were maintained for additional 30 min in the simultaneous presence of 500 μM ATP. Cell survival was measured 120 min post-treatment and data represent means ± SEM ($n = 6$). Statistical differences were verified by one-way analysis of variance and compared with the ATP value. MK801: hydrogen maleate; CPP: 3-(2-carboxypiperazin-4-yl) propyl-1-phosphonic acid; AP5: 2-amino-5-phosphonopentanoic acid; HA966: 3-amino-1-hydroxy-2-pyrrolidone.

1998) are also induced by extracellular purines in CGN, leading us to hypothesise a cross talk between these two neurotransmitter receptors. Alternatively, excitatory amino acids and purines might interact at a signal transduction level.

Whereas previous work has established that extracellular ATP is a signalling molecule for CGN (Volonté and Merlo, 1996; Volonté et al., 1999; Cavaliere et al., 2001a,b), the present study now proves that ATP can mediate death in dissociated primary cerebellar granule or striatal neurons and in hippocampal organotypic cultures. While abundant evidence accumulated over the years has demonstrated that different cell populations are killed by sustained exposure to high extracellular ATP (Zoeteweij et al., 1996; Ferrari et al., 1997; Schulze-Lohoff et al., 1998; Nihei et al., 2000), similar results had been lacking so far for neurons. In addition, we have shown here that the toxic insult mediated by ATP doesn't occur passively but, rather, CGN play an active role in causing their own loss. This is evident by the observed transcription/translation dependency and extensive nuclear damage occurring with cell death. Nevertheless, features of necrosis such as cellular swelling and extracellular release of the cytosolic enzyme lactic dehydrogenase are simultaneously observed. We conclude that CGN die in response to ATP through a particular combination of events, which include especially direct modulation of various P2 receptor subclasses.

In an attempt to discover the contribution of P2 receptor subtypes (single or more likely receptor combinations) to ATP-evoked cell death, our molecular-pharmacological results would exclude $P2Y_2$ (also UTP-activated), $P2Y_3$ (not RT-PCR-detected, UTP-activated), $P2Y_4$ (also UTP-activated), $P2Y_6$ (not ATP-activated), and $P2Y_8$ (not expressed in mammalian cells). Similarly, we would rule out $P2X_4$ (suramin- and PPADS-insensitive) and $P2X_{6,8}$ (not RT-PCR-detected, suramin-insensitive). On the other hand, $P2Y_1$, $P2Y_{11}$, $P2X_1$, $P2X_2$, $P2X_3$ and $P2X_5$ remain candidates. The involvement of $P2X_7$ (that should be inhibited by ATP-2′, 3′-dialdehyde and KN62, Murgia et al., 1992; Humphreys et al., 1998) is still debated, whereas adenosine receptors are clearly excluded.

In conclusion, due to the massive extracellular release of purine nucleotides and nucleosides occurring also dur-

ing in vivo insults (Lutz and Kabler, 1997; Phillis et al., 1993), our data indicate that extracellular ATP can be included among the concomitant causes of neurodegenerative events targeting the CNS. However, our knowledge of the regulatory mechanisms and signalling cascades underlying this complex molecular degeneration program is limited for the moment. Hopefully, a more precise understanding of these mechanisms will identify novel targets for the development of drugs reducing cell death associated with many neuropathological states.

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