The $P2X_7$ Receptor Sustains the Growth of Human Neuroblastoma Cells through a Substance P–Dependent Mechanism

Lizzia Raffaghello,[1] Paola Chiozzi,[2] Simonetta Falzoni,[2] Francesco Di Virgilio,[2] and Vito Pistoia[1]

[1]Laboratory of Oncology, Giannina Gaslini Institute, Genova, Italy and [2]Department of Experimental and Diagnostic Medicine, Section of General Pathology, and Interdisciplinary Center for the Study of Inflammation, University of Ferrara, Ferrara, Italy

Abstract

$P2X_7$ is a receptor for extracellular nucleotides expressed by different normal cell types. $P2X_7$ triggering may result in stimulation of cell proliferation or induction of apoptosis depending on the level of activation. $P2X_7$ expression and function in B-cell chronic lymphocytic leukemia has been shown to correlate with disease severity. Here, we have asked the question of whether $P2X_7$ is expressed and functional in neuroblastoma, a pediatric tumor of neuroectodermal origin. $P2X_7$ was detected both in primary neuroblastoma tumors and in neuroblastoma cell lines. In the latter cells, $P2X_7$ stimulation by ATP was found to trigger (*a*) increased intracellular calcium fluxes, (*b*) plasma membrane depolarization, and (*c*) formation of a nonselective plasma membrane permeable pore. In contrast to the usual response typically observed in the majority of cell types, $P2X_7$ *in vitro* stimulation did not induce caspase-3 activation or apoptosis of neuroblastoma cells but rather supported their proliferation. Growth stimulation was partially due to substance P release from nucleotide-activated neuroblastoma cells. Therefore, neuroblastoma cells seem to have molded $P2X_7$ function to their advantage in two ways (i.e., by silencing $P2X_7$ proapoptotic activity and by coupling $P2X_7$ stimulation to release of locally acting trophic factors). (Cancer Res 2006; 66(2): 907-14)

Introduction

Nucleotides are an ubiquitous family of extracellular signaling molecules that exert different effects through the interaction with plasma membrane receptors named P2 receptors (1). According to the molecular structure, P2 receptors are subdivided into P2Y and P2X families (2). The former were identified as seven-membrane–spanning G protein-coupled receptors and the latter as multimeric ligand-gated plasma membrane ion channels that mediate transmembrane cation fluxes (3, 4). Originally characterized in excitable cells, P2 receptors have been later identified in many cells of widely different origin (5, 6). One of the P2X subtypes, $P2X_7$, has attracted much interest for its unique behavior. Whereas transient stimulation with ATP causes the opening of the channel and the concomitant Ca^{2+} and Na^+ influx and K^+ efflux, sustained stimulation with ATP induces the appearance of a large, nonselective membrane pore, which permeabilizes the cells to larger molecules (7). This event results in cell apoptosis through the activation of metalloproteinases and caspases (8). However, $P2X_7$ activation can also induce lymphocyte proliferation, because transfection of $P2X_7$ cDNA increases the proliferative capacity of lymphoid cells (9). Additional events induced by long-term $P2X_7$ activation include the release of interleukin (IL)-1β, plasma membrane blebbing, and macrophage fusion (10–12).

The $P2X_7$ receptor is expressed in normal hematopoietic cells, such as lymphocytes, dendritic cells, and macrophages (7). Recent studies showed that $P2X_7$ is differentially expressed in neoplastic cells from patients with indolent B-cell chronic lymphocytic leukemia (B-CLL) versus progressive B-CLL courses (13). In these latter cells, ATP had a strong cytotoxic effect (13). Because no information is available about $P2X_7$ expression and function in tumor cells of different origin, we have here investigated these issues in human neuroblastoma cells.

Neuroblastoma is the most common extracranial tumor of childhood, derived from the sympathetic nervous system (14). Whereas stage I and II tumors are localized and well differentiated and can usually be cured by surgical resection, only patients with stage III and IV tumors present regional and disseminated disease, respectively, often characterized by low response to conventional treatments and poor prognosis (14).

In this study, we show that $P2X_7$ is expressed in neuroblastoma primary tumors and cell lines and that functional activation of this receptor by ATP is coupled to massive increase in cytosolic calcium, membrane depolarization, and uptake of larger hydrophilic molecules. Furthermore, we show that $P2X_7$ stimulation by ATP induces early morphologic changes without signs of apoptosis and late increase of cell proliferation mediated by substance P secretion.

Materials and Methods

Immunohistochemical staining of primary neuroblastoma tissues. This investigation was done after approval by a local institutional review board.

The criteria used for diagnosis and evaluation of disease extension have been reported elsewhere (15). Briefly, diagnosis was based on histologic grounds or on bone marrow infiltration by tumor cells, usually associated with elevated urinary catecholamine excretion.

The disease was staged according to the criteria of the International Neuroblastoma Staging System (15). They include the measurement of primary tumor size with ultrasonography and/or computed tomography, a bone marrow study by at least one aspirate, a skeletal study by plain X-ray survey and/or technetium 99^m DPM scintigraphy, and the measurement of urinary vanillylmandelic and homovanillic acids and of serum lactate dehydrogenase (15).

The primary neuroblastic tumors used in this study were obtained from patients at diagnosis before the implementation of any therapy.

Tissue samples were fixed in 20% buffered formalin, routinely processed, and embedded in paraffin. Immunohistochemical staining of tissue sections was done using the Envision System Horseradish Peroxidase (HRP) Mouse (DAKO, Glostrup, Denmark) as described previously (16). Briefly, 5-μm-thick sections were cut from formalin-fixed, paraffin-embedded blocks,

Note: L. Raffaghello and P. Chiozzi contributed equally as first authors to the work and F. Di Virgilio and V. Pistoia contributed equally as last authors to the work.

Requests for reprints: Lizzia Raffaghello, Laboratory of Oncology, Giannina Gaslini Institute, Largo Gerolamo Gaslini 5, Genova, Italy 16147. Phone: 39-10-5636342; Fax: 39-10-3779820; E-mail: lizziaraffaghello@ospedale-gaslini.ge.it.

doi:10.1158/0008-5472.CAN-05-3185

deparaffinized with xylene, and rehydrated by passages through decreasing concentrations of ethanol (100-80%). Endogenous peroxidase activity was blocked by a 30-minute incubation at room temperature with methanol containing 3% H_2O_2. Tissue sections were then incubated at 98°C for 40 minutes in citrate buffer (pH 6.0) for antigen retrieval (ChemMate, DAKO). After rinsing in Optimax Wash Buffer (Menarini Diagnostics, Firenze, Italy), tissue sections were incubated overnight at 4°C with optimal amounts of rabbit polyclonal anti-$P2X_7$ antibody raised against the synthetic peptide corresponding to the last 20 amino acids of the $P2X_7$ protein and kindly provided by Dr. Gary Buell (Serono, Geneva, Switzerland) or rabbit IgG isotype control (Southern Biotechnology Associates, Inc., Birmingham, AL) as negative controls. Tissue sections were washed twice in Optimax Wash Buffer and incubated for 30 minutes at room temperature with DAKO Envision System HRP Mouse. After washing in Optimax Wash Buffer, peroxidase activity was detected by incubating tissue sections for 6 to 10 minutes at room temperature with DAKO Liquid 3,3′-Diaminobenzidine Substrate Chromogen System (DAKO). Tissue sections were counterstained with Mayer's hematoxylin (Sigma, St. Louis, MO).

Serial tissue sections were stained with NB84 (DAKO) and CD45 monoclonal antibodies (mAb; UCHL1, DAKO) that detect neuroblasts and cells of hematopoietic origin, respectively. Areas containing at least 80% to 90% neuroblasts were selected for the analysis of $P2X_7$ expression. To this end, these areas were first inspected at low magnification and then carefully analyzed at higher magnification (×63).The percentage of stained tumor cells in each lesion was evaluated independently by two investigators. The variation between the results obtained by these investigators was <10%. Results were scored as negative or positive when the percentage of stained tumor cells in each microscopic area was <25% or >25%, respectively. The inclusion of each tumor sample in one of the above scores was based on the score of the microscopic area containing the highest percentage of mAb-positive neuroblasts.

Cell lines and flow cytometry. The ACN, GI-ME-N, HTLA-230, GI-CA-N, LAN-5, LAN-1, SK-N-BE-2, and SH-SY-5Y human neuroblastoma cell lines and the human acute monocytic leukemia THP1 cell line were cultured in RPMI 1640 (Sigma) supplemented with L-glutamine, penicillin/streptomycin, nonessential amino acids, and 10% fetal bovine serum (FBS; Sigma; complete medium). Phycoerythrin (PE)–conjugated AffiniPure $F(ab)_2$ fragments of sheep anti-rabbit IgG antibodies were purchased from Serotec Ltd. (Oxford, United Kingdom). Intracellular staining of cell lines was done as described. Briefly, cells were washed thrice with PBS (Sigma) containing 1% FBS (staining buffer) and fixed with 2% paraformaldehyde at room temperature for 20 minutes. Then, cells were washed twice with staining buffer and incubated in permeabilization buffer (PBS, 1% FBS, and 0.1% saponin; Sigma) for 30 minutes at room temperature. Cells (5×10^5 per tube) were next incubated with the primary rabbit polyclonal anti-$P2X_7$ antibody or rabbit IgG isotype control for 30 minutes at room temperature and then washed twice with permeabilization buffer and incubated with PE-conjugated $F(ab')_2$ fragments of sheep anti-rabbit IgG antibodies for 20 minutes at room temperature. Cells were then washed twice in permeabilization buffer and resuspended in staining buffer before being analyzed by flow cytometry using a FACScan instrument (BD Biosciences, San Jose, CA). CellQuest software (BD Biosciences) was used for data analysis. The results of flow cytometry experiments are expressed as percentage of positive cells.

Immunofluorescence. Human neuroblastoma cell lines were seeded on glass slides, washed with PBS, and fixed with paraformaldehyde (2% in PBS). After washing with PBS, the slides were incubated with 100 mmol/L ammonium chloride for 20 minutes at 4°C. Then, the cells were permeabilized with Triton X-100 (0.1% in PBS) and blocked with human serum for 20 minutes at 4°C. After washing with PBS, the cells were incubated for 1 hour with polyclonal anti-$P2X_7$ antibody overnight at 4°C. Cells were then rinsed twice with PBS and incubated with a FITC-conjugated AffiniPure $F(ab')_2$ fragments of sheep anti-rabbit IgG antibodies for 30 minutes at 4°C. At the end of this incubation, slides were rinsed with PBS and analyzed with the fluorescence microscope (17).

Western blotting. Cells were lysed in lysis buffer containing 300 mmol/L sucrose, 1 mmol/L K_2HPO_4, 1 mmol/L $MgSO_4$, 5.5 mmol/L glucose, 20 mmol/L HEPES (pH 7.4), 1 mmol/L benzamidine, 1 mmol/L phenylmethylsulfonyl fluoride, 0.2 μg DNase, and 0.3 μg RNase by repeated freeze/thawing (three cycles). Proteins were separated on 7.5% SDS-polyacrylamide gel according to Laemmli and blotted overnight on nitrocellulose paper (Schleicher & Schuell Italia Srl, Legnano, Italy). The anti-$P2X_7$ antibody was used at a dilution of 1:100 in TBS buffer [10 mmol/L Tris-HCl, 150 mmol/L NaCl (pH 8.0)]. Secondary antibody was a anti-rabbit antibody conjugated to alkaline phosphatase (17).

Cytoplasmic free Ca^{2+} concentration measurements. Changes in the cytoplasmic free Ca^{2+} ($[Ca^{2+}]_i$) concentration were measured with the fluorescent indicator fura-2-AM using a LS50 Perkin-Elmer fluorometer (Perkin-Elmer, Beaconsfield, United Kingdom) equipped with temperature control and magnetic stirring. For fura-2-AM loading cells (1×10^7/mL) were incubated for 15 minutes in a saline solution, heretofore called standard saline, containing 125 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L $MgSO_4$, 1 mmol/L Na_2HPO_4, 5.5 mmol/L glucose, 5 mmol/L $NaHCO_3$, 1 mmol/L $CaCl_2$, and 20 mmol/L HEPES (pH 7.4 with NaOH) in the presence of 250 mmol/L sulfinpyrazone (Sigma-Aldrich, St. Louis, MO), that prevents spontaneous leakage of fura-2-AM. $[Ca^{2+}]_i$ measurements were done at 37°C in a low ionic strength solution containing 300 mmol/L sucrose, 1 mmol/L $MgCl_2$, 1 mmol/L K_2HPO_4, 5 mmol/L $KHCO_3$, 5.5 mmol/L glucose, 1 mmol/L $CaCl_2$, and 20 mmol/L HEPES (pH adjusted to 7.4 with KOH) at the 340/380 nm excitation wavelength ratio at an emission of 505 nm (18).

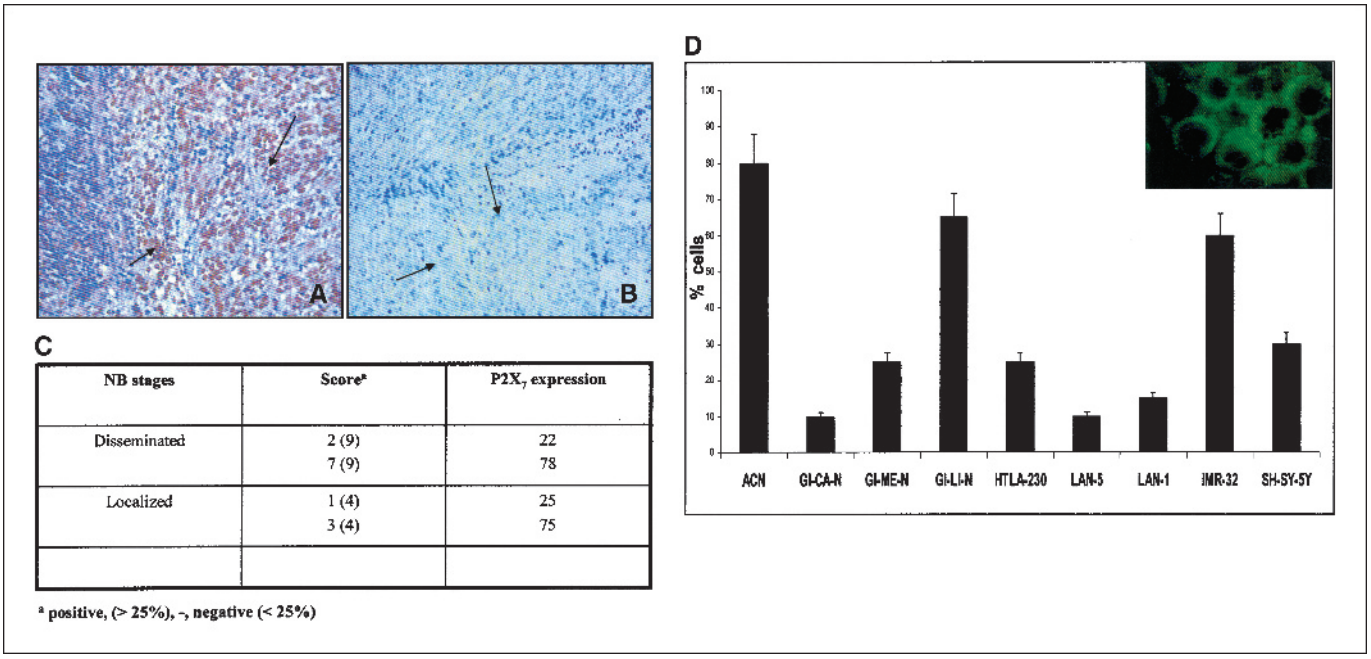
Semiquantitative measurement of plasma membrane potential. Changes in plasma membrane potential were measured in standard saline solution with the fluorescent dye bis-1,3-diethylthiobarbiturate trimethineoxonal (bisoxonol; Molecular Probes, Leiden, the Netherlands) at the wavelength pair 450/580 nm as described previously (18). Briefly, cells at a concentration of 100,000/mL were incubated at 37°C in a fluorimeter cuvette with magnetic stirring in the presence of 100 nmol/L bisoxonol. Depolarization triggered by nucleotide addition is expressed as percent change of maximal fluorescence increase caused by the addition of 60 mmol/L KCl.

Changes in plasma membrane permeability. $P2X_7$-mediated increases in plasma membrane permeability were measured by monitoring the uptake of the dye YO-PRO (Molecular Probes; ref. 17). Cell monolayers were incubated for 15 minutes at 37°C in standard saline containing 10 μmol/L YO-PRO in the presence or absence of various nucleotides. After several washings to remove the extracellular dye, cells were analyzed with an inverted fluorescence microscope (Olympus IMT-2, Olympus Optical Co. Ltd., Tokyo, Japan) equipped with a ×40 objective and fluorescein filter.

Morphologic changes and assessment of apoptosis. Human neuroblastoma cells were cultured in six-well plates (Costar, Cambridge, MA) for 1, 2, 4, 6, 12, and 24 hours in the absence or presence of ATP in the range of 0.5 to 5 mmol/L. For each time, the cells were observed under a contrast-phase microscope to evaluate morphologic changes and harvested by brief trypsinization. The proportion of apoptotic cells was assessed by flow cytometry using an Annexin V-FITC apoptosis kit according to the manufacturer's instruction (Bender MedSystems, Vienna, Austria). For measurement of caspase-3 activation, cells were seeded in 24-well plates at 37°C at a concentration of 200,000 per well in serum-free RPMI 1640 and stimulated with the various nucleotides for 4 hours. At the end of this incubation, cells were lysed and caspase-3 activation was measured fluorimetrically with a fluorescence-based kit as indicated by the manufacturer (Molecular Probes).

Assessment of proliferation. Cells were seeded at the concentration of 200,000 per well in 24-well plates in serum-free RPMI 1640 at 37°C. After 24 hours, cells were harvested and cell number was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test (Molecular Probes). Results were validated by cell counting. In some experiments, cells were also treated with increasing different concentrations of substance P (e.g., Fig. 5).

Assessment of IL-1β and substance P secretion. For IL-1β measurement, cells were seeded in 24-well plates at 37°C at a concentration of 200,000 per well, primed with bacterial lipopolysaccharide, and stimulated



C

NB stages	Score[a]	P2X$_7$ expression
Disseminated	2 (9) 7 (9)	22 78
Localized	1 (4) 3 (4)	25 75

[a] positive, (> 25%), –, negative (< 25%)

Figure 1. Expression of P2X$_7$ receptor in primary human neuroblastoma tumors and human neuroblastoma cell lines. Immunoperoxidase stainings of formalin-fixed, paraffin-embedded neuroblastoma primary tumors. *A,* representative staining for anti-P2X$_7$ receptor rabbit polyclonal antibody. *B,* negative control stained with an isotype and subclass-matched irrelevant antibody. *Arrows,* neuroblasts stained by the above mAbs. Original magnification, ×40. *C,* summary of the data obtained from immunohistochemical analysis of nine disseminated and four localized primary neuroblastoma (*NB*) tumors. *D,* expression of P2X$_7$ receptor by human neuroblastoma cell lines. The cell lines were stained with the rabbit polyclonal anti-P2X$_7$ receptor antibody and subsequently analyzed by flow cytometry. *Columns,* positive percentage cells from three experiments; *bars,* SD. *Inset,* surface localization of P2X$_7$ receptor in ACN cells by fluorescence microscopy.

with increasing nucleotide concentrations (100 μmol/L-5 mmol/L) for 30 minutes in serum-free RPMI 1640. At the end of this incubation time, supernatants were withdrawn and tested for IL-1β content by ELISA (R&D Systems, Minneapolis, MN). For substance P measurement, cell were incubated in serum-free RPMI 1640 and stimulated with the nucleotides for 2 hours. Substance P (Sigma) content of the supernatants was measured by ELISA (R&D Systems).

Results

P2X$_7$ receptor expression in primary human neuroblastoma tumors and cell lines. Paraffin-embedded tissue sections from newly diagnosed, Schwannian stroma poor, primary neuroblastoma were stained in the immunoperoxidase reaction with a rabbit polyclonal anti-P2X$_7$ antiserum. Figure 1*A* shows a representative

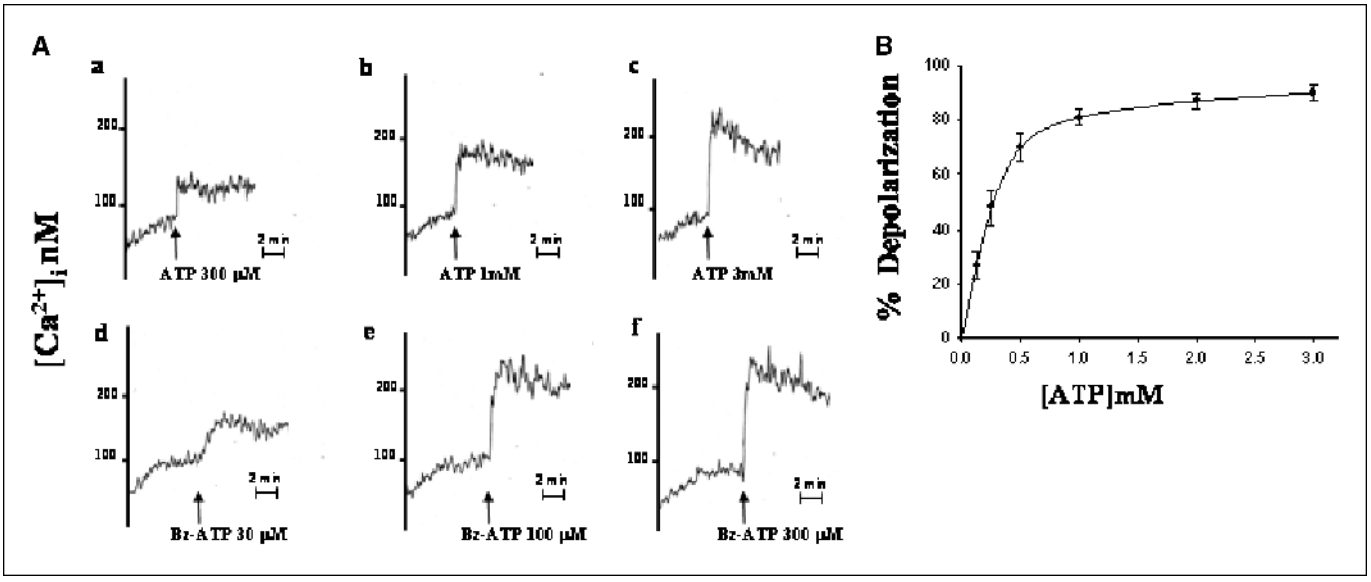


Figure 2. Effects of ATP and benzoyl-ATP on $[Ca^{2+}]_i$ and plasma membrane potential in ACN cells. *A,* changes in $[Ca^{2+}]_i$ triggered by ATP or benzoyl-ATP. ACN cells were incubated in Ca^{2+}-containing standard saline and challenged with increasing concentrations of the nucleotides. $[Ca^{2+}]_i$ was measured with the fluorescent indicator fura-2-AM as described in Materials and Methods. *B,* an ATP dose dependency of plasma membrane depolarization in Ca^{2+}-containing medium. Changes in plasma membrane potential were measured with bisoxonol as described in Materials and Methods. Representative of three experiments.

experiment in which the majority of tumor cells in every lesion displayed an intense staining pattern. In Fig. 1*B*, a negative control stained with an isotype and subclass-matched irrelevant antibody is shown. Figure 1*C* shows the proportion of neuroblastoma tumors testing positive for $P2X_7$ receptor expression. The majority of the tumors showed robust $P2X_7$ expression irrespective of the pattern of disease presentation (i.e., localized or metastatic).

Expression of $P2X_7$ protein was next investigated in a panel of human neuroblastoma cell lines by flow cytometry. $P2X_7$ receptor was detected in the cytosol of all human neuroblastoma cell lines (Fig. 1*D*). As shown in Fig. 1*D* (*inset*), immunofluorescence microscopy analysis of ACN cells revealed clear $P2X_7$ patches at the level of the plasma membrane as well as diffuse cytosolic staining. The same staining pattern was obtained in the THP1 control cell line (data not shown), in agreement with previous data showing that most THP1 cells have a large cytoplasmic reservoir of $P2X_7$ (11). These findings indicated that in both ACN and THP1 cells $P2X_7$ was expressed both in the transmembrane and in the cytoplasm.

Finally, expression of $P2X_7$ in human neuroblastoma cell lines was confirmed by Western blot experiments done with the same polyclonal antiserum used for fluorescence-activated cell sorting analysis and immunofluorescence microscopy (data not shown).

The above data show unambiguously that the $P2X_7$ protein is expressed in both primary neuroblastoma tumors and neuroblastoma cell lines.

Functional characterization of $P2X_7$ in human neuroblastoma cell lines. We next investigated the functional responses coupled to activation of $P2X_7$ in human neuroblastoma cell lines. Despite the presence of sulfinpyrazone, ACN cells showed a large spontaneous leakage of fura-2-AM, a finding not unusual in tumors cells (Fig. 2*A*). Both ATP and the ATP pharmacologic analogue benzoyl-ATP, which are known to induce Ca^{2+} fluxes in many cells expressing the $P2X_7$ (5, 19), triggered a massive $[Ca^{2+}]_i$ increase. The dose-dependent effect of ATP and benzoyl-ATP was biphasic, suggesting the activation of two different classes of P2 receptors, one with high affinity (ATP EC_{50} in the 10 μmol/L range, presumably P2Y) and one with low affinity (ATP EC_{50} of ~1 mmol/L, presumably $P2X_7$). As mentioned, $P2X_7$ has two functional activation states: as a cation selective channel and, on sustained stimulation, as a nonselective pore (7). One of the earliest changes due to $P2X_7$ activation is the collapse of plasma membrane potential due to the large Na^+ influx. As shown in Fig. 2*B*, ATP caused a large plasma membrane depolarization in ACN cells, with an EC_{50} of ~200 μmol/L. The more potent $P2X_7$ agonist benzoyl-ATP also caused plasma membrane potential collapse, but with a lower EC_{50} (30 μmol/L; data not shown).

The hallmark of $P2X_7$ activation is an ATP-dependent permeabilization of the plasma membrane to large molecules, including the fluorescent dyes lucifer yellow, YO-PRO, and ethidium bromide (7, 20). In Fig. 3, we show the effect on YO-PRO uptake of treatment of ACN cells with 1 mmol/L ATP for 15 minutes. As shown in Fig. 3*A*, in the absence of the nucleotide, cells showed negligible dye uptake mainly due to fluid phase endocytosis. ATP caused a massive YO-PRO uptake by most cells (Fig. 3*C*). Quite interestingly, and in agreement with observations done in other cell types (11), ATP stimulation also caused plasma membrane blebbing and vesicle release into the extracellular space. Similar results were obtained in other neuroblastoma cell lines (data not shown).

The Ca^{2+}/calmodulin-dependent protein kinase II inhibitor KN-62 has been proposed as a selective and potent antagonist for the human $P2X_7$ (21). As shown in Fig. 3*E*, treatment with this inhibitor strongly reduced ATP-triggered YO-PRO uptake, a strong proof for the direct involvement of the $P2X_7$ in ATP-dependent plasma membrane permeabilization in ACN cells.

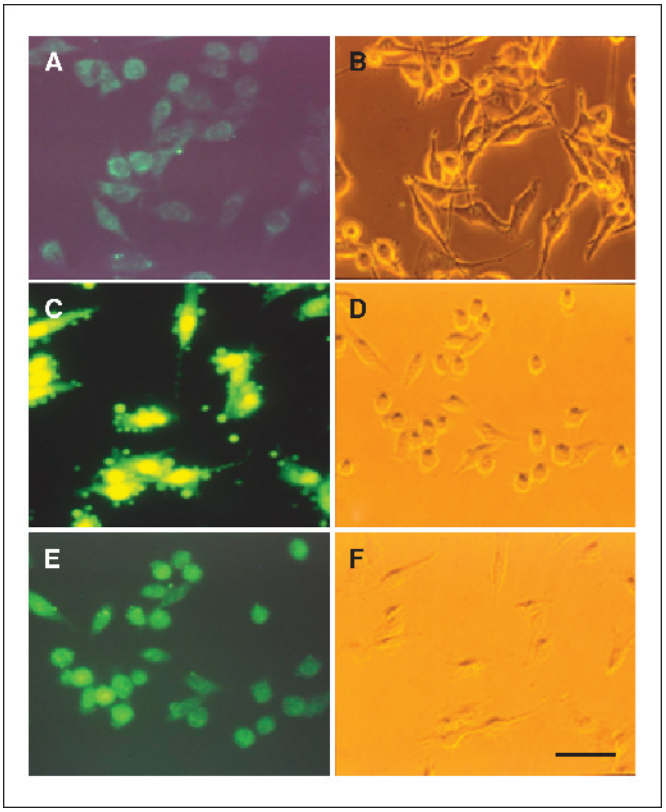


Figure 3. ATP-dependent YO-PRO uptake in ACN cells. ACN cells were incubated for 15 minutes at 37°C in serum-free RPMI 1640 in the presence of 10 μmol/L YO-PRO and in the absence (*A* and *B*; control) or presence (*C-F*) of 1 mmol/L ATP. *E* and *F*, $P2X_7$ receptor antagonist KN-62 (500 nmol/L) was also added to the incubation medium. *A, C*, and *E*, fluorescence microscopy. *B, D*, and *F*, phase-contrast microscopy. Bar, 20 μm. Representative of three experiments.

Cell proliferation and apoptosis induced by $P2X_7$ activation in human neuroblastoma cell lines. Activation of the $P2X_7$ caused striking and dose-dependent morphologic alterations in neuroblastoma cell lines. The earliest shape change was loss of neurites and cell shrinkage, already detectable in ACN cells at a concentration of 0.5 mmol/L ATP (Fig. 4*A, b*). Such morphologic alterations were not observed in untreated ACN cells (Fig. 4*A, a*).

At higher concentrations, plasma membrane blebbing also occurred in most ACN cells (Fig. 4*A, c* and *d*). Very interestingly, all these changes were fully reversible on removal of ATP, and in contrast to most other cell types expressing $P2X_7$ (22), no signs of apoptosis or necrosis were detected at any time as assessed by Annexin V staining (data not shown). Similar morphologic changes were observed in different neuroblastoma cell lines treated with ATP (data not shown).

Lack of $P2X_7$-dependent cytotoxicity was further supported by the inability of ATP or benzoyl-ATP to activate caspase-3 in neuroblastoma cells (Fig. 4*B*). In contrast, control rat mesangial cells, well known to have a strong $P2X_7$-dependent caspase-3 activity (23), were dose-dependently stimulated on incubation with ATP or benzoyl-ATP (Fig. 4*B*).

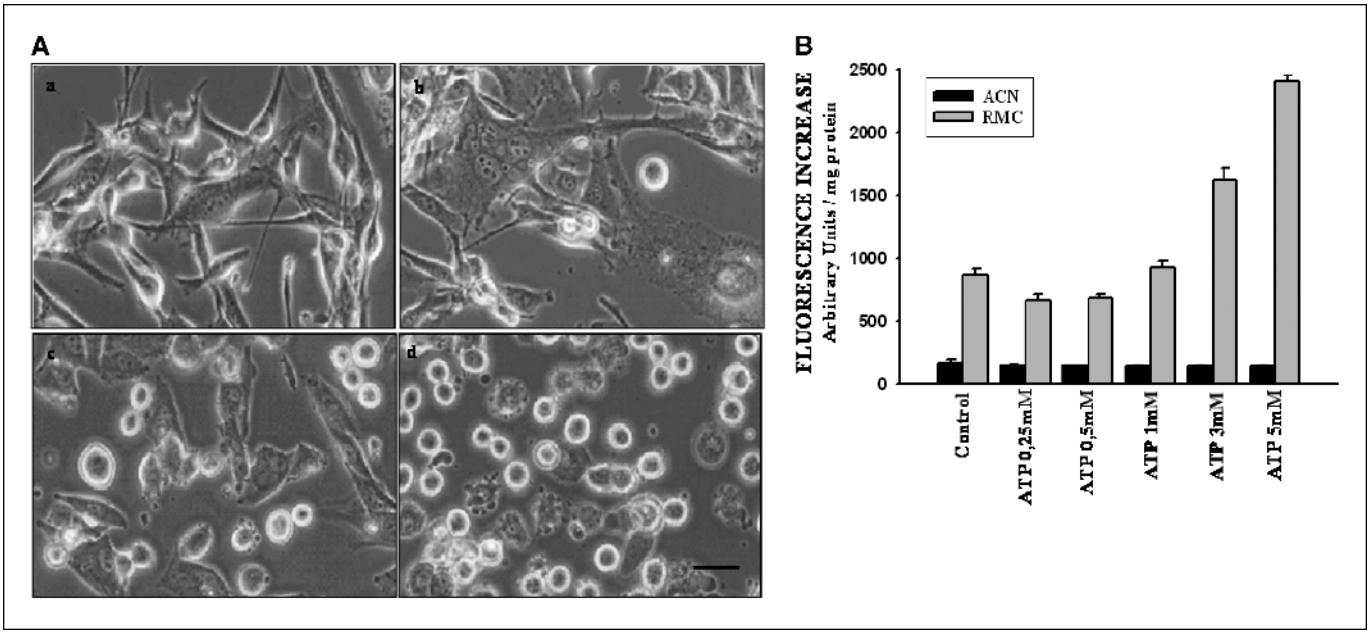


Figure 4. $P2X_7$ stimulation causes neuroblastoma cell rounding and swelling without activation of caspase-3. *A,* ACN cells were incubated at 37°C in serum-free RPMI 1640 in absence (*a*) or presence of 0.5 (*b*), 2 (*c*), or 5 (*d*) mmol/L ATP for 1 hour and analyzed by phase-contrast microscopy. Bar, 20 μm. Representative of three experiments. *B,* ACN or rat mesangial cells (*RMC*) were incubated at 37°C in serum-free RPMI 1640 as described in Materials and Methods and stimulated with increasing ATP concentrations. At the end of the incubation, cells were lysed and caspase-3 activity was measured by ELISA. *Columns,* mean of three experiments; *bars,* SD.

Lack of an overt cytotoxic effect in neuroblastoma cells due to $P2X_7$ activation was surprising, because this receptor/pore is a well-known trigger of cell death. However, in some cell types, $P2X_7$ can act as a growth-promoting receptor (9, 13). To investigate this alternative function, we monitored neuroblastoma cell proliferation by the MTT assay following exposure to different stimuli. As shown in Fig. 5, ATP at the optimal concentration of 0.5 mmol/L promoted ACN cell proliferation. However, the proliferative effect was not confined to ATP, but other nucleotides (e.g., benzoyl-ATP, UTP, ADP, and AMP) as well as adenosine promoted growth of ACN cells in the absence of serum. The two nucleotide-hydrolyzing enzymes apyrase and hexokinase also promoted growth in the absence of added exogenous nucleotides. This indicates that the mechanism whereby extracellular nucleotides stimulate growth of ACN cells is complex, involving activation of P2X, P2Y, and P1 receptors. In addition, the growth-promoting effect of apyrase and hexokinase suggests that ACN cells continuously release into the pericellular space ATP that can be then

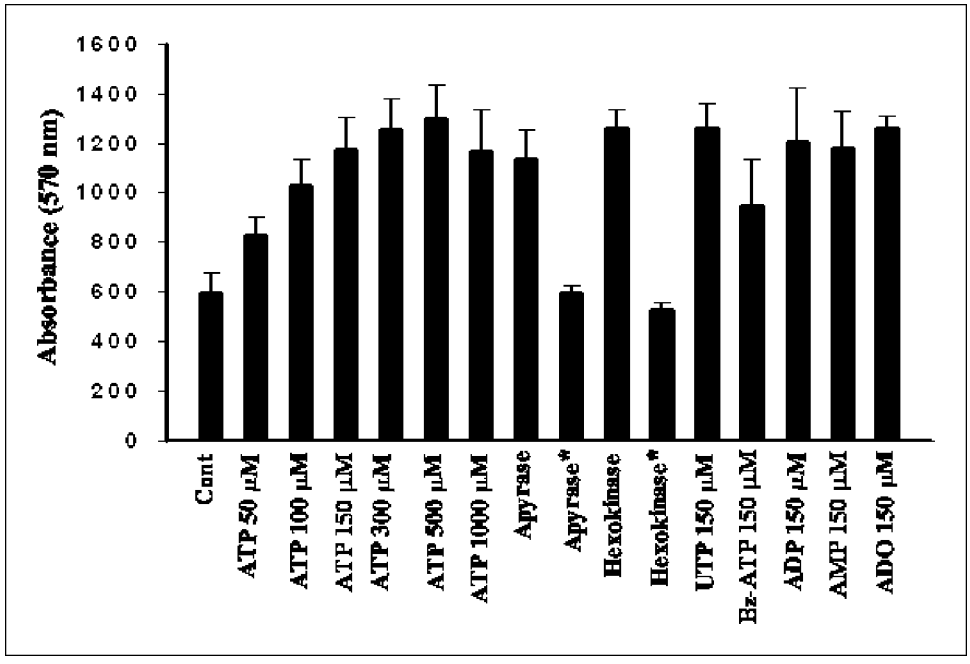


Figure 5. Extracellular nucleotides and nucleosides cause proliferation of ACN cells. ACN cells were incubated in the absence or presence of various nucleotides or nucleosides at the indicated concentrations for 24 hours. In some experiments, the effect of native or heat-inactivated (*asterisk*) apyrase or hexokinase was also tested. Proliferation was evaluated by MTT assay and direct cell counting. *Columns,* mean of three experiments; *bars,* SD.

converted to adenosine. In keeping with this interpretation, inactivation by boiling of the two enzymes suppressed growth stimulation (Fig. 5). Measurement of ATP secretion by the standard soluble luciferase assay or by a recently developed plasma membrane–targeted chimeric luciferase showed that ACN maintain a resting pericellular ATP level in the 100 to 200 nmol/L range that increases up to 100 μmol/L on stimulation (24). Similar results were obtained with other neuroblastoma cell lines (data not shown).

Substance P released on P2 receptor stimulation promotes neuroblastoma cell proliferation. Although direct activation of P2 and P1 receptors by nucleotides and adenosine can by itself support growth, it cannot be excluded that additional factors with growth-promoting activity are released by ACN cells on stimulation with nucleotides. In fact, it is well documented that P2Y and $P2X_7$ activation can induce release of trophic factors, such as tumor necrosis factor-α, IL-1β, and IL-6, in different cell types (17, 22, 25). Therefore, we investigated whether stimulation with extracellular nucleotides can cause secretion of IL-1β and substance P, two molecules that can be produced by human neuroblastoma cell lines and regulate their growth (26, 27). We were unable to detect IL-1β release from ACN cells under several experimental conditions (data not shown). On the contrary, a large release of substance P was triggered by ATP, benzoyl-ATP, and UTP (Fig. 6*A*). Benzoyl-ATP was a better agonist that the other two nucleotides tested, suggesting a main role for the $P2X_7$ receptor (Fig. 6*A*). Substance P secreted by ACN cells on stimulation was in the 150 to 400 pg/mL range; thus, we investigated whether this amount was sufficient to stimulate ACN cell growth. As shown in Fig. 6*B*, addition of substance P in the 100 to 300 pg/mL range caused almost a doubling of the ACN cell population. Similar results were obtained with other neuroblastoma cell lines (data not shown). These results show that P2 receptor activation in neuroblastoma cells triggers release of powerful paracrine/autocrine growth-promoting factors besides directly stimulating proliferation.

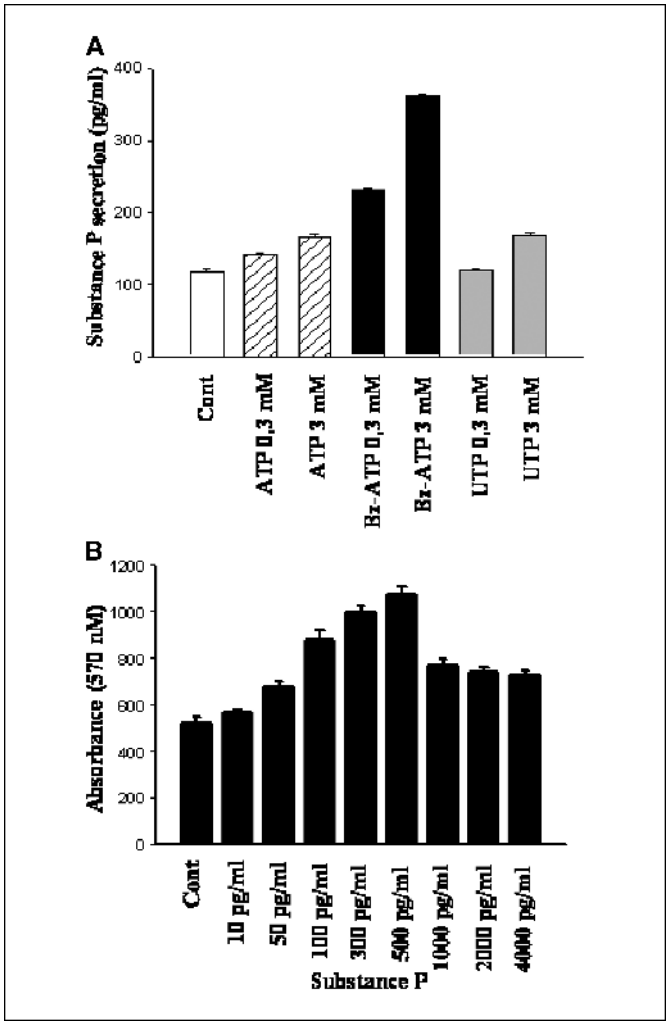


Figure 6. Substance P is secreted on stimulation with extracellular nucleotides and triggers proliferation of ACN cells. *A,* ACN cells were incubated as described in Materials and Methods and challenged with the different nucleotides at the indicated concentrations. *B,* ACN cells lines were incubated as described in Materials and Methods in the absence or presence of increasing substance P concentrations for 24 hours. Proliferation was assessed by MTT assay and direct cell counting. *Columns,* mean of three experiments; *bars,* SD.

Discussion

Extracellular nucleotides have now acquired a full status of mediators of cell-to-cell communication (28–31).

The complexity of this extracellular messenger system is highlighted by the number of P2 receptor subtypes thus far identified and by their ubiquitous distribution. Furthermore, different nucleotide selectivity and affinity endow this system with a remarkable plasticity.

Among P2 receptors, $P2X_7$ is one of the most intriguing, owing to its ability to mediate plasma membrane permeabilization to low molecular mass aqueous solutes by an as yet poorly understood process. This permeabilization process is likely due to the opening of a nonselective pore that is generally thought to coincide with the receptor itself (7, 32) but that according to other investigators might be a separate molecular entity (33).

Several tumors have been shown to express $P2X_7$ at unusually high levels (13, 34–38). In some cases (i.e., breast and prostate cancer), it was suggested that the $P2X_7$ might be nonfunctional (36, 37), but no data were presented to support this claim. In all other instances, tumor cells expressed fully functional $P2X_7$.

In this study, we show for the first time that human neuroblastoma cells from either primary tumors or cell lines expressed $P2X_7$ receptors. The functionality of these receptors was shown by different assays, such as ligand-induced changes in free intracellular calcium concentrations, membrane depolarization, and uptake of the YO-PRO dye.

Among P2X receptors, only the $P2X_7$ subtype is able to cause a collapse of plasma membrane permeability barrier most likely through its extended COOH-terminal cytoplasmic tail that allows interaction with several intracellular proteins and possibly organelles (39). The physiologic meaning of this phenomenon is basically unknown, but because plasma membrane pore formation is a common trigger of cell death, as epitomized by complement and several bacterial toxins, it is widely assumed that $P2X_7$ is a cytotoxic receptor.

We found that, on incubation with ATP or benzoyl-ATP, neuroblastoma cells underwent distinctive morphologic changes, including loss of neurites, swelling, and shrinkage, all suggestive of an ongoing apoptotic process. However, these phenomena were transient and no apoptosis of neuroblastoma cells was detected by either Annexin V assay or caspase-3 activation. These results showed that the latter cells were refractory to $P2X_7$ ligand-induced

apoptosis and suggested that this behavior of tumor cells could represent a survival strategy.

Additional functions of $P2X_7$ unrelated to cytotoxicity have been identified, such as triggering of cell proliferation (9, 40), cytokine release (41–43), and transcription factor activation (44, 45).

Two reports from some of us (P.C., S.F., and F.D.V.) have shown that overexpression of the $P2X_7$ provides a growth/survival advantage under limiting culture conditions (i.e., in the absence of serum-derived factors; refs. 9, 13).

Although the biochemical basis of this effect is as yet poorly understood, available evidence suggests that a small but significant elevation in cytoplasmic and intramitochondrial Ca^{2+} levels plays a crucial role (43). Thus, a tumor would be in a very favorable position if it could silence the negative (death inducing) responses linked to $P2X_7$ activation and keep only the positive (growth supporting).

We tested this hypothesis by investigating proliferation of neuroblastoma cells induced by $P2X_7$ ligands (i.e., ATP and the specific agonist benzoyl-ATP). The experiments done with the latter ligand allowed to conclude unambiguously that selective $P2X_7$ triggering resulted into an enhancement of neuroblastoma cell proliferation. At least part of this effect was found to depend on the induced release of substance P that stimulated in a dose-dependent manner neuroblastoma cell growth. Substance P is a tachykinine family member that has an important role in inflammation and has been shown recently to promote neuroblastoma growth (27).

Assignment of a functional role to receptors for extracellular nucleotides implies by necessity the presence of these molecules in the extracellular milieu. Therefore, an obvious, but as yet unproven, assumption is that in the tumor microenvironment there is enough extracellular ATP to activate P2 receptors, including the low-affinity $P2X_7$. We have measured the extracellular ATP concentrations by the standard luciferin/luciferase assay or by a plasma membrane–targeted chimeric luciferase and found that ACN cells keep a steady ATP concentration in the incubation medium of 100 to 200 nmol/L (24),[3] a value ~10-fold higher than that usually found in culture supernatants from different cell types (46). With the availability of more sophisticated techniques, it is becoming clear that ATP is released into the extracellular milieu not only at sites of tissue damage but also in the perilesional regions (47). Whether a high extracellular ATP concentration is also a feature of the neuroblastoma microenvironment is an open question that is currently the subject of active investigation.

In conclusion, neuroblastoma cells seem to have molded $P2X_7$ receptor function to their advantage in two ways: on the one hand, they have silenced $P2X_7$ cytotoxic activity by blocking its ability to turn on caspase-3 and, on the other, they have exploited $P2X_7$ capacity to trigger the release of locally acting trophic factors, like substance P. Based on these findings, it is tempting to speculate that targeted ATP inactivation in the tumor microenvironment may dampen tumor growth.

Acknowledgments

Received 9/8/2005; accepted 11/3/2005.

Grant support: Ministry of Education and Scientific Research (Cofin, Investment Fund for Basic Research), Italian Association for Cancer Research, institutional funds from the University of Ferrara, and funds from the Italian Ministry of Health.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[3] S. Falzoni and F. Di Virgilio, unpublished data.

References

1. Ralevic V, Burnstock G. Receptors for purines and pyrimidines. Pharmacol Rev 1998;50:413–92.
2. Abbracchio MP, Boeynaems JM, Barnard EA, et al. Characterization of the UDP-glucose receptor (re-named here the P2Y14 receptor) adds diversity to the P2Y receptor family. Trends Pharmacol Sci 2003;24:52–5.
3. von Kugelgen I, Wetter A. Molecular pharmacology of P2Y-receptors. Naunyn Schmiedebergs Arch Pharmacol 2000;362:310–23.
4. North RA. Molecular physiology of P2X receptors. Physiol Rev 2002;82:1013–67.
5. Di Virgilio F, Chiozzi P, Ferrari D, et al. Nucleotide receptors: an emerging family of regulatory molecules in blood cells. Blood 2001;97:587–600.
6. Hoebertz A, Arnett TR. Isolated osteoclast cultures. Methods Mol Med 2003;80:53–64.
7. Di Virgilio F. The P2Z purinoceptor: an intriguing role in immunity, inflammation and cell death. Immunol Today 1995;16:524–8.
8. Ferrari D, Los M, Bauer MK, Vandenabeele P, Wesselborg S, Schulze-Osthoff K. P2Z purinoreceptor ligation induces activation of caspases with distinct roles in apoptotic and necrotic alterations of cell death. FEBS Lett 1999;447:71–5.
9. Baricordi OR, Melchiorri L, Adinolfi E, et al. Increased proliferation rate of lymphoid cells transfected with the P2X(7) ATP receptor. J Biol Chem 1999;274:33206–8.
10. Solle M, Labasi J, Perregaux DG, et al. Altered cytokine production in mice lacking P2X(7) receptors. J Biol Chem 2001;276:125–32.
11. Morelli A, Chiozzi P, Chiesa A, et al. Extracellular ATP causes ROCK I-dependent bleb formation in $P2X_7$-transfected HEK293 cells. Mol Biol Cell 2003;14:2655–64.
12. Chiozzi P, Sanz JM, Ferrari D, et al. Spontaneous cell fusion in macrophage cultures expressing high levels of the P2Z/$P2X_7$ receptor. J Cell Biol 1997;138:697–706.
13. Adinolfi E, Melchiorri L, Falzoni S, et al. $P2X_7$ receptor expression in evolutive and indolent forms of chronic B lymphocytic leukemia. Blood 2002;99:706–8.
14. Brodeur GM. Neuroblastoma: biological insights into a clinical enigma. Nat Rev Cancer 2003;3:203–16.
15. Brodeur GM, Seeger RC, Barrett A, et al. International criteria for diagnosis, staging, and response to treatment in patients with neuroblastoma. J Clin Oncol 1988;6:1874–81.
16. Raffaghello L, Prigione I, Bocca P, et al. Multiple defects of the antigen-processing machinery components in human neuroblastoma: immunotherapeutic implications. Oncogene 2005;24:4634–44.
17. Solini A, Chiozzi P, Morelli A, Fellin R, Di Virgilio F. Human primary fibroblasts *in vitro* express a purinergic $P2X_7$ receptor coupled to ion fluxes, microvesicle formation and IL-6 release. J Cell Sci 1999;112:297–305.
18. Falzoni S, Munerati M, Ferrari D, Spisani S, Moretti S, Di Virgilio F. The purinergic P2Z receptor of human macrophage cells. Characterization and possible physiological role. J Clin Invest 1995;95:1207–16.
19. Humphreys BD, Dubyak GR. Induction of the P2Z/$P2X_7$ nucleotide receptor and associated phospholipase D activity by lipopolysaccharide and IFN-γ in the human THP-1 monocytic cell line. J Immunol 1996;157:5627–37.
20. Steinberg TH, Newman AS, Swanson JA, Silverstein SC. ATP4- permeabilizes the plasma membrane of mouse macrophages to fluorescent dyes. J Biol Chem 1987;262:8884–8.
21. Gargett CE, Wiley JS. The isoquinoline derivative KN-62 a potent antagonist of the P2Z-receptor of human lymphocytes. Br J Pharmacol 1997;120:1483–90.
22. Bulanova E, Budagian V, Orinska Z, et al. Extracellular ATP induces cytokine expression and apoptosis through $P2X_7$ receptor in murine mast cells. J Immunol 2005;174:3880–90.
23. Solini A, Iacobini C, Ricci C, et al. Purinergic modulation of mesangial extracellular matrix production: role in diabetic and other glomerular diseases. Kidney Int 2005;67:875–85.
24. Pellegatti P, Falzoni S, Pinton P, Rizzuto R, Di Virgilio F. A novel recombinant plasma membrane-targeted luciferase reveals a new pathway for ATP secretion. Mol Biol Cell 2005;16:3659–65.
25. Idzko M, Panther E, Stratz C, et al. The serotoninergic receptors of human dendritic cells: identification and coupling to cytokine release. J Immunol 2004;172:6011–9.
26. Corasaniti MT, Bilotta A, Strongoli MC, Navarra M, Bagetta G, Di Renzo G. HIV-1 coat protein gp120 stimulates interleukin-1β secretion from human neuroblastoma cells: evidence for a role in the mechanism of cell death. Br J Pharmacol 2001;134:1344–50.
27. Munoz M, Rosso M, Perez A, Covenas R, Rosso R, Zamarriego C, Piruat JI. The NK1 receptor is involved in the antitumoural action of L-733,060 and in the mitogenic action of substance P on neuroblastoma and glioma cell lines. Neuropeptides 2005;39:427–32.
28. Burnstock G. Introduction: P2 receptors. Curr Top Med Chem 2004;4:793–803.
29. Erlinge D. Extracellular ATP: a central player in the regulation of vascular smooth muscle phenotype. Focus on "Dual role of PKA in phenotype modulation of vascular smooth muscle cells by extracellular ATP." Am J Physiol Cell Physiol 2004;287:C260–2.

30. Di Virgilio F. Dr. Jekyll/Mr. Hyde: the dual role of extracellular ATP. J Auton Nerv Syst 2000;81:59–63.

31. Holzer AM, Granstein RD. Role of extracellular adenosine triphosphate in human skin. J Cutan Med Surg 2004;8:90–6.

32. Surprenant A, Rassendren F, Kawashima E, North RA, Buell G. The cytolytic P2Z receptor for extracellular ATP identified as a P2X receptor ($P2X_7$). Science 1996;272: 735–8.

33. Schilling WP, Wasylyna T, Dubyak GR, Humphreys BD, Sinkins WG. Maitotoxin and P2Z/P2X(7) purinergic receptor stimulation activate a common cytolytic pore. Am J Physiol 1999;277:C766–76.

34. Zhang XJ, Zheng GG, Ma XT, et al. Expression of $P2X_7$ in human hematopoietic cell lines and leukemia patients. Leuk Res 2004;28:1313–22.

35. Wang Q, Wang L, Feng YH, Li X, Zeng R, Gorodeski GI. $P2X_7$ receptor-mediated apoptosis of human cervical epithelial cells. Am J Physiol Cell Physiol 2004;287: C1349–58.

36. Slater M, Danieletto S, Gidley-Baird A, Teh LC, Barden JA. Early prostate cancer detected using expression of non-functional cytolytic $P2X_7$ receptors. Histopathology 2004;44:206–15.

37. Slater M, Danieletto S, Pooley M, Cheng Teh L, Gidley-Baird A, Barden JA. Differentiation between cancerous and normal hyperplastic lobules in breast lesions. Breast Cancer Res Treat 2004;83:1–10.

38. Greig AV, Linge C, Cambrey A, Burnstock G. Purinergic receptors are part of a signaling system for keratinocyte proliferation, differentiation, and apoptosis in human fetal epidermis. J Invest Dermatol 2003;121:1145–9.

39. Kim M, Jiang LH, Wilson HL, North RA, Surprenant A. Proteomic and functional evidence for a $P2X_7$ receptor signalling complex. EMBO J 2001;20:6347–58.

40. Baricordi OR, Ferrari D, Melchiorri L, et al. An ATP-activated channel is involved in mitogenic stimulation of human T lymphocytes. Blood 1996;87:682–90.

41. Perregaux DG, McNiff P, Laliberte R, Conklyn M, Gabel CA. ATP acts as an agonist to promote stimulus-induced secretion of IL-1β and IL-18 in human blood. J Immunol 2000;165:4615–23.

42. Ferrari D, Chiozzi P, Falzoni S, Hanau S, Di Virgilio F. Purinergic modulation of interleukin-1β release from microglial cells stimulated with bacterial endotoxin. J Exp Med 1997;185:579–82.

43. Adinolfi E, Callegari MG, Ferrari D, et al. Basal activation of the $P2X_7$ ATP receptor elevates mitochondrial calcium and potential, increases cellular ATP levels, and promotes serum-independent growth. Mol Biol Cell 2005;16:3260–72.

44. Ferrari D, Stroh C, Schulze-Osthoff K. $P2X_7$/P2Z purinoreceptor-mediated activation of transcription factor NFAT in microglial cells. J Biol Chem 1999;274: 13205–10.

45. Ferrari D, Wesselborg S, Bauer MK, Schulze-Osthoff K. Extracellular ATP activates transcription factor NF-κB through the P2Z purinoreceptor by selectively targeting NF-κB p65. J Cell Biol 1997;139:1635–43.

46. Beigi R, Kobatake E, Aizawa M, Dubyak GR. Detection of local ATP release from activated platelets using cell surface-attached firefly luciferase. Am J Physiol 1999;276:C267–78.

47. Wang X, Arcuino G, Takano T, et al. $P2X_7$ receptor inhibition improves recovery after spinal cord injury. Nat Med 2004;10:821–7.