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ACTIVATION OF Src/KINASE/PHOSPHOLIPASE C/MITOGEN-ACTIVATED PROTEIN KINASE AND INDUCTION OF NEURITE EXPRESSION BY ATP, INDEPENDENT OF NERVE GROWTH FACTOR

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Abstract—Extracellular ATP has been reported to potentiate the neurite outgrowth induced by nerve growth factor. In the present study the neurotrophic effect of ATP and other nucleotides was examined in mouse neuroblastoma neuro2a cells which lack nerve growth factor receptor. Exposure of neuro2a cells to ATP resulted in a dramatic increase in neurite bearing cells as compared with untreated control cells. Experiments performed with purinergic receptor agonists and antagonists suggest that the ATP stimulates neurite outgrowth via P2 receptors. Neurite outgrowth was completely blocked by P2 receptor antagonist suramin whereas the P1 receptor antagonist CGS15943 was ineffective. P1 receptor agonist 5′-(*N*-ethylcarboxamido)adenosine failed to induce neurite outgrowth. The potency order of different P2 receptor agonists was ATP=ATPγS>ADP≫2Me-S-ATP. It was insensitive to UTP and antagonist pyridoxal phosphate-6-azo (benzene-2,4-disulfonic acid) suggesting the involvement of P2Y11 receptor in the observed neuritogenic effect. The signaling pathway leading to ATP-induced neuritogenesis was investigated. The neuritogenic effect of ATP is independent of rise in intracellular Ca^{2+} as pharmacological profile of neuritogenic P2Y receptor does not match with that of P2Y2 receptor associated with $[Ca^{2+}]_i$ signaling cascade. Exposure of cells to ATP caused activation of Src kinase, phospholipase Cγ and extracellular signal-regulated kinases ERK1/2. Mitogen-activated protein kinase (MAPK) inhibitor U0126 drastically reduced the number of neurite bearing cells in ATP-treated cultures implying that the neurotrophic effect of ATP is mediated by MAPK. Our results demonstrate that ATP can stimulate neurite outgrowth independent of other neurotrophic factors and can be an effective trophic agent.

Key words: ATP, P2Y receptor, MAP kinase, neurite outgrowth, neuro2a cells.

Trophic factors play a crucial role in neuronal plasticity during development and regeneration of damaged nervous system. The most widely known neurotrophic factor nerve growth factor (NGF) has been suggested as a therapeutic agent for neurodegenerative disorders, such as Alzheimer's disease (Lad et al., 2003). However, there are neuronal cell types in the mammalian nervous system which are insensitive to NGF, therefore other neurotrophic agents are of enormous interest as potential therapeutic agents. Recent reports indicate a trophic role for extracellular ATP in the development and maintenance of the nervous system and its response to injury or diseases (Neary et al., 1996; Rathbone et al., 1999; Franke and Illes, 2006). ATP induces proliferation of astrocytes, microglia and brain capillary endothelial cells (Rathbone et al., 1992; Brambilla and Abbracchio, 2001). Rathbone et al. (1999) proposed that purines may interact with neurons or neuronal precursors, eliciting neuritogenesis, maintenance of existing neurites and enhance survival. Some earlier studies have shown that ATP and GTP potentiate the NGF-induced neurite outgrowth in PC 12 cells (Gysbers et al., 2000; D'Ambrosi et al., 2000; Behrsing and Vulliet, 2004). ATP alone enhanced the cell survival but was not capable of inducing neuritogenesis.

Extracellular ATP acts via purinergic receptors of P1 and P2 class, which exhibit distinct sensitivity to different nucleotides. The potency order of P1 receptors is adenosine>AMP>ADP>ATP whereas the P2 receptors show opposite potency i.e. ATP>ADP>AMP>adenosine (Fredholm et al., 1994). The P2 receptors were originally classified into P2X and P2Y receptors based on pharmacological studies on the activity of ATP analogues and antagonists. P2X receptors are ligand-gated ionotropic receptors that are principally activated by ATP, with the other nucleotides having very low potency (North, 2002). P2Y receptors belong to the G protein-coupled receptor family. Within each receptor class several receptor subtypes have been cloned and pharmacologically characterized. To date four adenosine P1 receptors: A1, A2A, A2B and A3, seven P2X receptor subunits designated $P2X_{1-7}$ and eight mammalian G protein-coupled P2 receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$) have been cloned and pharmacologically characterized (Ralevic and Burnstock, 1998).

Purinergic receptors activate a myriad of signaling cascades. Extracellular signal regulated kinase (ERK) is a key member of the mitogen-activated protein kinase (MAPK) family and is a crucial mediator of trophic effects including neuronal plasticity (Bonni et al., 1999; Riccio et al., 1999). P2 receptor-mediated MAPK activation has been demonstrated in astrocytes and PC12 cells. Inhibition of ERK cascade prevented the ATP-induced mitogenesis in rat and human fetal astrocytes (Neary et al., 1999). Stimula-

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Abbreviations: BSA, bovine serum albumin; ERK, extracellular signal-regulated kinase; GPCR, G-protein-coupled receptor; MEK, MAP kinase/ERK kinase kinase; MAPK, mitogen-activated protein kinase; NECA, 5′-(*N*-ethylcarboxamido)adenosine; NGF, nerve growth factor; PBS, phosphate-buffered saline; pERK1/2, phosphorylated extracellular signal-regulated kinase 1/2; PLCγ, phospholipase Cγ; PMSF, phenylmethylsulphonyl fluoride; PPADS, pyridoxal phosphate-6-azo (benzene-2,4-disulfonic acid); SD, somal diameter.

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tion of PC12 cells by ATP/UTP activated MAPK ERK1/2 through a Ca^{2+} and PKC dependent process (Soltoff, 1998; Swanson et al., 1998). Recently (Behrsing and Vulliet, 2004) have shown that elevation of MAPK activity by ATP enhances the NGF-induced neurite expression and P2 receptors were suggested to be the most likely mediator of this effect.

In the present study we have investigated the neurotrophic effects of ATP and other nucleotides in neuro2a cells, which lack NGF receptors (Matta et al., 1986). Neuro2a cells exhibit neuritogenesis in response to a variety of agents viz. retinoic acid, gangliosides, dibutyryl cAMP, and serum deprivation. Different signaling mechanisms have been reported to be associated with the differentiation process. Ganglioside GM1-induced differentiation is initiated by Ca^{2+} influx (Fang et al., 2000). Activation of Src kinase is associated with GM3-induced neuritogenesis (Prinetti et al., 1999). Increased phosphorylation of EGFR, ERK1/2 and Akt mediates the differentiation of cells upon serum withdrawal (Evangelopoulos et al., 2005). Recently it has been reported that the retinoic acid and dibutyryl cAMP induced neurite outgrowth is mediated by up-regulation of Rho-family guanine nucleotide exchange factor GEFT (Bryan et al., 2006). Here we report that ATP can directly induce neurite expression in NGF insensitive neuro2a cells by activation of MAPKs ERK1/2 via P2 receptor activation.

EXPERIMENTAL PROCEDURES

Materials

Rosewell Park Memorial Institute (RPMI 1640) medium, bovine serum albumin (BSA), nucleotides ATP, ADP, AMP, UTP, adenosine, ATP analogues ATPγS, $\alpha\beta$ methyl ATP, 2 methyl-S-ATP, and adenosine analogues 5′-(*N*-ethylcarboxamido)adenosine (NECA), CGS15943, suramin, pyridoxal phosphate-6-azo (benzene-2,4-disulfonic acid) (PPADS), U0126, MRS 2179, neomycin sulfate, aprotinin, leupeptin, pepstatin A, phenylmethylsulphonyl fluoride (PMSF), sodium orthovanadate, dithiothreitol (DTT), ammonium persulphate (APS), acrylamide, were obtained from Sigma Aldrich Foreign Holding Co., Bangalore, India. Fetal calf serum was from Gibco-BRL, Carlsbad, CA, USA. Phosphate buffered saline (PBS) was procured from Hi Media, Bombay, India.

The monoclonal antibodies against phospholipase Cγ (anti-PLCγ), Src kinase (anti-Yes) and phosphorylated tyrosine (PY20), anti-phosphotyrosine-coupled agarose and goat anti-mouse antibody–horseradish peroxidase conjugated (HRP-GAM) were procured from Transduction Laboratories, Lexington, KY, USA. Polyclonal antibodies against P2Y2 and P2Y11 receptors were obtained from Alomone Laboratories, Jerusalem, Israel. Alexa fluor-488 conjugated goat anti-rabbit antibody was from Molecular Probes, USA. SuperSignal® West Pico chemiluminescent substrate was purchased from Pierce, Rockford, IL, USA. All other chemicals were of analytical grade obtained from commercial sources.

Cell culture and measurement of neurite outgrowth

Neuro2a cells were obtained from National Center for Cell Sciences (Pune, India). Cells were grown in plastic culture flasks or on 30 mm Petri dishes (Falcon) in RPMI medium supplemented with 2 g/l sodium bicarbonate, 10% fetal calf serum, 50,000 U/l benzyl penicillin, and 3500 U/l streptomycin. Cells were incubated at 37 °C in an atmosphere of 5% CO_2 and 95% air. To investigate the neuritogenic effects of ATP and other agents the cells were plated at a density of 1×10^4 cells/cm^2 in 30 mm Petri dishes. After 24 h of growth, the medium was replaced with fresh serum free medium and desired amounts of ATP and/or other agents were added and further incubated for 48 h. In some experiments the cells were incubated with ATP for 1 h and subsequently they were maintained in serum free medium. Duplicate cultures were set up for each treatment. The neuritogenesis was assessed at 72 h of growth. Cultures were examined under a microscope (Olympus IX70). Phase contrast images were acquired and analyzed with TILL Vision software (TILL Photonics, Gmbh, Germany). Neurite outgrowth was quantified by determining the percentage of cells with neurites $\geq$1 or 2 somal diameter (SD) as described earlier by Ravichandra and Joshi (1999). Five different fields on the culture dishes containing 50–100 cells in each field were randomly selected and counted and the mean percentage of neurite bearing cells was obtained for each culture. Each treatment was repeated three to five times in duplicate cultures.

Immunocytochemistry and confocal microscopy

The immunofluorescence experiments were performed to detect the expression of P2Y receptors in neuro2a cells. Cells grown on polylysine-coated coverslips for 24 h were fixed for 30 min in 4% paraformaldehyde and permeabilized for 10 min in PBS containing 0.2% Triton-X. Nonspecific binding sites were blocked by pre-incubating the cells for 30 min with 10% BSA in PBS. This was followed by an incubation with primary antibodies against P2Y2 receptor (1:50) and P2Y11 (1:100) for 2 h at room temperature. Subsequently, the cultures were washed thrice in PBS and incubated with Alexa fluor-488-conjugated goat anti-rabbit antibody (1:500) in 1% BSA in PBS for 30 min and washed three times in PBS. Control experiments were carried out with the primary antibodies being omitted from the staining protocol. The immunofluorescence was imaged by a confocal Laser microscope (Olympus FV 1000) using 40× W/IR objective lens. The images were taken using 488 nm argon ion laser.

Isolation of cell membrane

Whole cell membrane was prepared as described by Chen and Chen (1997) with some modifications. Cells grown in 75 cm^2 tissue culture flasks were washed twice with PBS and scraped with rubber policeman, and treated with prechilled hypotonic lysis buffer (10 mM Tris pH 8.0, 5 mM KCl, 1 mM dithreitol, 1 mM EGTA, 100 μg/ml PMSF, 1 μg/ml each of aprotinin, leupeptin, and pepstatin A, 100 μM sodium orthovanadate) for 15 min at 4 °C. The lysate was homogenized on ice using dounce homogenizer with Teflon pestle. The homogenate was centrifuged at $1000\times g$ for 15 min in a refrigerated centrifuge (Hettich, Germany) to remove the nuclear fraction. The post–nuclear fraction was centrifuged at $11{,}000\times g$ at 4 °C for 15 min. The membrane pellet was resuspended in 500 μl of the hypotonic lysis buffer with the protease inhibitors mentioned above and stored at −50 °C until use.

Immunoprecipitation of tyrosine-phosphorylated proteins

Cells grown in 75 cm^2 culture flasks were washed with PBS and exposed to stimulatory agents for 15 min at room temperature in presence of 1 mM $CaCl_2$ or absence of Ca^{2+} with 1 mM EGTA. The medium was immediately aspirated and membrane fractions were prepared. Fifty micrograms of the treated and untreated membrane fraction was vortexed thoroughly with RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.25% sodium deoxycholate, 1 mM EDTA, 1 mM PMSF, 1 mM NaF, 1 mM sodium