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P19 EMBRYONAL CARCINOMA CELLS AS *IN VITRO* MODEL FOR STUDYING PURINERGIC RECEPTOR EXPRESSION AND MODULATION OF *N*-METHYL-D-ASPARTATE–GLUTAMATE AND ACETYLCHOLINE RECEPTORS DURING NEURONAL DIFFERENTIATION

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Abstract—The *in vitro* differentiation of P19 murine embryonal carcinoma cells to neurons resembles developmental stages which are encountered during neuronal development. Three days following induction to neuronal differentiation by retinoic acid, most cells of the P19 population lost expression of the stage specific embryonic antigen (SSEA-1) and expressed the neural progenitor cell specific antigen nestin. Beginning from day 4 of differentiation nestin expression was down-regulated, and expression of neuron-specific enolase as marker of differentiated neurons increased. The molecular mechanisms underlying neuronal differentiation are poorly understood. We have characterized the participation of purinergic ionotropic (P2X) and metabotropic (P2Y) receptors at mRNA transcription and protein levels as well as ATP-induced Ca^{2+} transients during neuronal differentiation of P19 cells. Gene and protein expression of $P2X_2$, $P2X_6$, $P2Y_2$, and $P2Y_6$ receptors increased during the course of differentiation, whereas $P2X_3$, $P2X_4$, $P2Y_1$ and $P2Y_4$ receptor expression was high in embryonic P19 cells and then decreased following induction of P19 cells to differentiation. $P2X_1$ receptor protein expression was only detected on days 2 and 4 of differentiation. Although $P2X_5$ and $P2X_7$ mRNA transcription was present, no protein expression for this receptor subunit could be detected throughout the differentiation process. In undifferentiated cells, mainly ionotropic P2X receptors contributed to the ATP-induced Ca^{2+}-response. In neuronal-differentiated P19 cells, the ATP-induced Ca^{2+}-response was increased and the metabotropic component predominated. Purinergic receptor function is implicated to participate in neuronal maturation, as cholinergic and glutamate–*N*-methyl-D-aspartate (NMDA) induced calcium responses were affected when cells were differentiated in the presence of purinergic receptor antagonists pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), suramin or reactive blue-2. Our data suggest that inhibition of $P2Y_1$ and possibly $P2X_2$ receptors led to a loss of NMDA receptor activity whereas blockade of possibly $P2X_2$ and $P2Y_2$ purinergic receptors during neuronal differentiation of P19 mouse led to inhibition of cholinergic receptor responses.

Key words: purinergic receptors, P19 embryonal carcinoma cells, neuronal differentiation, maturation of synapses, intrinsic modulation of cholinergic and glutamate–NMDA receptor activity, ATP.

Brain development is one of the most important morphogenetic events occurring in the embryo and is a complex process involving cell proliferation and differentiation as well as tissue organization into a specific architecture. The understanding of the mechanism of neuronal differentiation lies in the determination of how a small number of tissue-restricted transcription factors can establish a complex pattern of expression of developmental and tissue-specific genes. Previous studies have identified several growth factors and neurotransmitters that influence the proliferation of embryonic or adult mammalian neural stem cells (NSCs) (Gage et al., 1995; Cameron et al., 1998; Erlandsson et al., 2001), but little is known about the signaling molecules that determine the self-renewal capacity of NSCs. Purinergic receptors, activated by ATP and other nucleotides, have been attributed to several functions including neurotransmission and neuromodulation in synapses (Zimmermann, 1994; Neary et al., 1996; Franke and Illes, 2006). However, purinergic receptor expression and activity are not only found in the adult brain but also in early stages of embryonic development (Franke and Illes, 2006) suggesting that these receptors may play a role in embryogenesis. ATP binds to and activates ligand-gated ionotropic purinergic ionotropic receptors (P2X) and G-protein-coupled metabotropic purinergic metabotropic receptors (P2Y) (Abbracchio and Burnstock, 1994), thereby inducing a variety of cellular functions, including differentiation and proliferation. Nowadays seven subtypes of ionotropic ($P2X_1$–$P2X_7$) and eight subtypes ($P2Y_{1,2,4,6,11–14}$) of metabotropic purinoreceptors have been identified by molecular cloning. Purinergic receptors have been shown to be essential for the proliferation and migration of oligodendrocytes progenitors in the CNS (Agresti et al., 2005),

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Abbreviations: $[Ca^{2+}]_i$, intracellular free calcium concentration; CCh, carbamoylcholine; DMEM, Dulbecco's modified Eagle's medium; EB, embryonic body; EC, embryonal carcinoma; EGTA, ethylene glycol tetraacetic acid; FBS, fetal bovine serum; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; NEL, neuron-specific enolase; NF-160, medium-molecular weight neurofilaments; NF-200, high-molecular weight neurofilaments; NMDA, *N*-methyl-D-aspartate; NSC, neural stem cells; PB, phosphate buffer; PC12, pheochromocytoma cells; PPADS, pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid; P2X, purinergic ionotropic receptors; P2Y, purinergic metabotropic receptors; RA, retinoic acid; RNAi, RNA interference; SSEA-1, stage specific embryonic antigen.

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control of hippocampus neurogenesis (Shukla et al., 2005) and direction of migration events as demonstrated by trophic roles of extracellular ATP on astrocytes and neurons (Abbracchio et al., 1994; Neary et al., 1996).

Complex developmental processes can be studied in a simplified environment using pluripotent cells as *in vitro* models which can differentiate into a defined phenotype. The P19 murine embryonal carcinoma (EC) cell line is such an *in vitro* model that can be differentiated into various cell types by cellular aggregation in presence of the differentiating agent retinoic acid (RA). The differentiation of this cell line emulates the molecular and morphological events occurring during early embryonic development (McBurney et al., 1982). Following treatment with RA, P19 cells differentiate into various neuroectodermal derivatives (McBurney et al., 1982) and express neuronal markers, including high-molecular weight neurofilaments (NF-200), β-3-tubulin, neuron-specific enolase (NEL) (Niemann and Schaller, 1996; Martins et al., 2005), glutamate receptors (MacPherson et al., 1997), GABA receptors (Reynolds et al., 1996) and proteins specific for cholinergic neurons (Jones-Villeneuve et al., 1982; Cauley et al., 1996; Parnas et al., 1998) indicating that the establishment of a neuronal phenotype proceeds with similar molecular patterning during neuronal differentiation of P19 cells and *in vivo* development (Bayer and Altman, 1995; Wiese et al., 2004; Wu and Chow, 2005). The expression of neurotransmitters and their receptors becomes upregulated during precursor stages and synapse formation in cortical and subcortical regions (Nguyen et al., 2001). This time-regulated expression and activity of neurotransmitter receptors can also be observed during neuronal differentiation of P19 cells; for instance, cholinergic, NMDA (*N*-methyl-D-aspartate), endothelin-B, and kinin-B2 receptors are already present in neural precursor cell stages and become fully functional in P19 neurons (Martins et al., 2005; Monge et al., 1995; Ulrich and Majumder, 2006).

We have used the P19 cell line as *in vitro* model for studying expression and activity of purinergic P2X and P2Y receptors during differentiation from embryonic to neural precursor stages and finally to neurons expressing functional NMDA-glutamate receptors and muscarinic and nicotinic acetylcholine receptors. We have verified the participation of purinergic receptor function by analyzing the fate of neuronal differentiation in the presence of the purinergic receptor antagonists pyridoxalphosphate-6-azophenyl-2′,4′-disulphonic acid (PPADS), reactive blue-2 and suramin. We show that loss of NMDA or cholinergic receptor responses in differentiated neurons is due to inhibition of $P2Y_1$ and possibly $P2X_2$ or $P2Y_2$ and possibly $P2X_2$ receptor activity, respectively.

EXPERIMENTAL PROCEDURES

Materials

If not otherwise noticed, all reagents were purchased from Sigma (St. Louis, MO, USA) in highest available purity. The P19 murine EC cell line was kindly provided by Dr. H. C. Schaller, Center for Molecular Neurobiology, Hamburg, Germany. $P2X_1$- (PC378) and $P2X_3$ (PC411)-specific antibodies were purchased from Oncogene Research Products (Cambridge, MA, USA). $P2X_4$ (sc-15187), $P2X_6$ (sc-15197), $P2Y_1$ (sc-15203), $P2Y_4$ (sc-17634) receptor-specific antibodies were from Santa Cruz Biotechnologies (Heidelberg, Germany). $P2X_7$ receptor-specific antibodies (506107) and anti-β-tubulin mouse mAb (DM1B) were obtained from Calbiochem (San Diego, CA, USA). $P2X_2$ (ab5244), $P2X_5$ (ab9226), $P2Y_2$ (ab5931 and ab5816) and $P2Y_6$ (ab5715) receptor-specific antibodies were from Chemicon International (Temecula, CA, USA).

Mice (*Mus musculus*, Balbi-C strain) were obtained from the Animal Facility, Institute of Chemistry, University of São Paulo. The protocols for keeping and killing of these animals were approved by the Bioethics Committee of the Chemistry Institute, Universidade de São Paulo, Brazil, and are in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize the number of animals used and their suffering.

In vitro differentiation of P19 cells into neurons

P19 mouse EC cells were cultured and differentiated to neurons as described previously (Tárnok and Ulrich, 2001; Martins et al., 2005). Briefly, P19 cell cultures were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS, Cultilab, Campinas, Brazil), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 2 mM sodium pyruvate. For induction of neuronal differentiation, 5×10^5 P19 cells/ml in defined medium, containing DMEM medium supplemented with 2 mM glutamine, 2 mM sodium pyruvate, 2.4 mg/ml sodium bicarbonate, 5 μg/ml insulin, 30 μg/ml human apo-transferrin, 20 μM ethanolamine, 30 nM sodium selenite, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM Hepes, pH 7.2, were treated with 1 μM "all-trans" RA and plated into bacterial dishes previously coated with 0.5% agarose, to avoid adhesion of the cell culture to plastic surfaces. After 2 days of culture in suspension in the presence of RA, P19 cells formed embryonic body stages (EBs). EBs were collected from suspension cultures and replated in adherent culture flasks in DMEM medium with 10% FBS for 48 h. The serum-containing medium was replaced with defined medium on day 4, followed by four more days of culture until neuronal maturation was completed as determined by neuron-specific protein expression (neurofilament-200 and β-3-tubulin) according to Martins et al., 2005. Glial cells were eliminated from differentiating neuron cultures by addition of cytosine-arabinoside (5 μg/ml) 2 days before cell removal.

Immunohistochemistry

Cells at a density of 5×10^5 cells/ml were grown on glass coverslips, fixed in 2% paraformaldehyde for 10 min and dehydrated on a platform heated at 37 °C. Incubations were performed as described below for the immunohistochemistry procedure. Goat or rabbit antibodies raised against purinergic receptor subtypes (see Materials) and mouse anti-medium-molecular weight neurofilaments (NF-160) and mouse anti-NF-200 antibodies were used at 1:150, 1:500 and 1:500 dilutions, respectively. Goat anti-rabbit and goat anti-mouse IgG were used at 1:200 dilutions. Conjugated secondary antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA, USA). The cells were collected in phosphate buffer (PB) and incubated overnight with goat anti-P2X or P2Y receptor antibodies (1:150) in the presence of 0.3% Triton X-100 and 5% normal goat serum. After washing with PB, cells were incubated with biotinylated goat anti-rabbit antibodies at 1:200 dilution during 1 h. Following washing with PB cells were incubated with an avidin–biotin complex for 1 h. Peroxidase activity was detected using diaminobenzidine as a chromogen and H_2O_2 (Adhikari et al., 2006). Negative controls were obtained by repeating the procedure above in the absence of the primary antibody. Images were collected from an optical microscope equipped with the Nikon Digital Camera DXM1200F and analyzed