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Mechanism of acetylcholine-induced calcium signaling during neuronal differentiation of P19 embryonal carcinoma cells *in vitro*

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Abstract

Muscarinic (mAChRs) and nicotinic acetylcholine receptors (nAChRs) are involved in various physiological processes, including neuronal development. We provide evidence for expression of functional nicotinic and muscarinic receptors during differentiation of P19 carcinoma embryonic cells, as an *in vitro* model of early neurogenesis. We have detected expression and activity of α_2–α_7, β_2, β_4 nAChR and M1–M5 mAChR subtypes during neuronal differentiation. Nicotinic α_3 and β_2 mRNA transcription was induced by addition of retinoic acid to P19 cells. Gene expression of α_2, α_4–α_7, β_4 nAChR subunits decreased during initial differentiation and increased again when P19 cells underwent final maturation. Receptor response in terms of nicotinic agonist-evoked Ca^{2+} flux was observed in embryonic and neuronal-differentiated cells. Muscarinic receptor response, merely present in undifferentiated P19 cells, increased during neuronal differentiation. The nAChR-induced elevation of intracellular calcium ($[Ca^{2+}]_i$) response in undifferentiated cells was due to Ca^{2+} influx. In differentiated P19 neurons the nAChR-induced $[Ca^{2+}]_i$ response was reduced following pretreatment with ryanodine, while the mAChR-induced response was unaffected indicating the contribution of Ca^{2+} release from ryanodine-sensitive stores to nAChR- but not mAChR-mediated Ca^{2+} responses. The presence of functional nAChRs in embryonic cells suggests that these receptors are involved in triggering Ca^{2+} waves during initial neuronal differentiation.

Keywords: Neuronal differentiation; P19 embryonal carcinoma cells; Acetylcholine receptors; Ryanodine receptors; IP_3 receptors; Ca^{2+} signaling

1. Introduction

The development of the nervous system is one of the most important morphogenetic events occurring in the embryo. This process is accompanied by cell proliferation and differentiation as well as by tissue organization into a specific architecture. Although the specific molecular pathways that drive these events remain unresolved, it is widely believed that proliferation and differentiation programs of the neural progenitors require the interaction of extrinsic and intrinsic signals. While the function of growth factors in controlling neuronal differentiation is well documented, there also is increasing persuasive evidence for a role of neurotransmitters and their respective receptors in this process [1,2]. Many neurotransmitters are already present in the brain prior to axonogenesis and synaptogenesis, raising the possibility that they may mediate non-classical signaling. One such neurotransmitter is acetylcholine (ACh), whose biological actions are mediated by both nicotinic (nAChRs) and muscarinic receptors (mAChRs). Neuronal nAChRs are heterogeneous, with at least six α (α_2–α_7) and three β (β_2–β_4) subunits (reviewed in [3]). Nicotinic receptor subunits are among the first membrane proteins to appear during central nervous system (CNS) development, and their initial expression does not

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depend on nerve contact [4]. Prior to innervation, nAChRs are diffusely distributed throughout the nerve fibre surface, while after innervation, nAChRs cluster at high concentrations at neuronal junctions [5–8]. Gene expression patterns and several functional properties of nAChRs change during differentiation and maturation of neurons [9,4,10,11]. Transcription of mRNAs coding for nAChR subunits and expression of α_3, α_4, α_7, β_2, and β_4 nicotinic receptor subunits in the developing rat from early embryonic through postnatal developmental stages [4] and during *in vitro* differentiation of a P19 mouse embryonic carcinoma (EC) cell line as a model for early development [12] point at a pivotal role during the onset of neurogenesis.

In contrast to nAChRs which directly participate in neurotransmission in mature neurons, muscarinic receptor function is predominately linked to modulation of synaptic activity. Five mAChR subtypes are present in the CNS, with the M5 subtype being exclusively expressed in the brain. These receptors activate multiple signaling pathways that are important for modulating neuronal excitability, synaptic plasticity and feedback regulation of ACh release [13]. Muscarinic receptor participation in neurogenesis has been suggested based on experimental evidence. For instance, treatment of neural stem and progenitor cells with ACh or its stable analogue carbamoylcholine led to increases in DNA synthesis and accelerated neurogenesis [14]. In another study a neuroblastoma cell line, which by itself does not synthesize ACh, was transfected with a construct coding for expression of choline acetyltransferase. The activation of ACh synthesis resulted in a higher expression of neuronal specific traits compared to untransfected control cells. The presence of mAChRs in these transfected cells indicates the presence of an autocrine loop that may be responsible for the advanced differentiation state ([15], reviewed in [16]).

In the present work, we provide evidence for the presence of functional nicotinic and muscarinic receptors during *in vitro* differentiation of P19 EC cells as a model of early embryogenesis. Neuronal differentiation of this cell line is believed to closely resemble the developmental stage of the embryonic ectoderm. The observation that in addition to differential cholinergic receptor expression, the expression of choline acetyltransferase and acetylcholine esterase with its splice variants is regulated throughout the course of differentiation [17,12,18,19], makes the P19 cell line a reliable model to study cholinergic receptor function during early neuronal development.

2. Materials and methods

2.1. Abbreviations used

4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methiodide; ACh, acetylcholine; CICR, Ca^{2+}-induced Ca^{2+} release; CPA, cyclopiazonic acid; DMEM, Dulbecco's modified Eagle's medium; EB, embryonic bodies; EM, extracellular medium; FBS, fetal bovine serum; F_{max}, maximal fluorescence; F_{min}, minimal fluorescence; IP_3, inositol-3-phosphate; mAChR, muscarinic acetylcholine receptors; MT3, green mamba *Dendroaspis angusticeps* toxin; nAChR, nicotinic acetylcholine receptors; NF, neurofilament; PBS, phosphate-buffered saline; PLC, phospholipase C; RA, "*all-trans*" retinoic acid; RT, reverse transcription; SERCA, sarcoplasmatic/endoplasmatic reticulum Ca^{2+}-ATPase; VOOC, voltage-operated Ca^{2+} channels.

2.2. Reagents

Unless otherwise indicated, all reagents were purchased from Sigma (St. Louis, MO). Primary monoclonal antibodies are from Covance (Covance Research Products, Denver, PA), rat anti-α_3 nAChR (MRT-611R), rat anti-α_4 nAChR (MRT-613R), rat anti-α_5 nAChR (MRT-623R), mouse anti-α_7 nAChR (MMS-627R) and rat anti-β_2 nAChR (MRT-639R) and appropriate secondary biotin conjugate antibodies were used at 1/200 dilutions (Santa Cruz Biotechnology, Heidelberg, Germany). The anti-α_2, -α_6 and -β_4 antibodies were a gift from Dr. Jon Lindstrom (Department of Neuroscience, University of Pennsylvania and Philadelphia, PA). Primers for RT-PCR reactions were synthesized by Integrated DNA Technologies, Coralville, IA.

2.3. Culture and neuronal differentiation of P19 cells

P19 murine embryonal carcinoma cells were grown in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% of final volume with fetal bovine serum (FBS) (Cultilab, Campinas, Brazil), 100 units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 2 mM sodium pyruvate, in a humidified incubator at 5% CO_2 and 37 °C. Neuronal differentiation was induced by addition of 1 μM RA. Formation of EB was induced by culturing P19 cells as cell suspension in bacterial culture dishes coated with 0.2% of final volume with agarose for 48 h at a density of 5×10^5 cells/ml in defined serum-free medium as described previously [20,21]. Completion of neuronal differentiation after 8 days of addition of RA was confirmed by immunostaining against neuron-specific proteins NF-200, β-3-tubulin, and neuron-specific enolase (data not shown). Differentiated P19 cell cultures were kept free from glial cells by treatment with 50 μg/ml cytosine arabinoside on day 6 of differentiation.

2.4. Reverse transcription (RT) and real-time PCR

Total RNA was isolated using the TRIzol reagent (Invitrogen) from embryonic P19 (day 0) and differentiating P19 cells on days 2–8 of differentiation. Contaminating DNA was removed by DNase I (Ambion Inc., Austin, TX) treatment and integrity of the isolated RNA was analyzed on a 2% ethidium bromide-stained agarose gel. Three micrograms of total RNA from days 0 to 8 of differentiation were used in