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Muscarinic receptor-activated signal transduction pathways involved in the neuritogenic effect of astrocytes in hippocampal neurons ☆

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ABSTRACT

Astrocytes have been shown to release factors that affect various aspects of neuronal development. We have previously shown that the acetylcholine analog carbachol, by activating muscarinic M_3 receptors in rat astrocytes, increases their ability to promote neuritogenesis in hippocampal neurons. This effect was mediated by an increased expression and release by astrocytes of several permissive factors, a most relevant of which was fibronectin. In the present study we investigated the signal transduction pathways involved in these effects of carbachol in astrocytes. Results show that multiple pathways are involved in the effects of carbachol on astrocyte-mediated increases in fibronectin expression and neuritogenesis. These include the phospholipase D pathway, leading to sequential activation of protein kinase C (PKC) ζ, p70S6 kinase and nuclear factor-κB; the phosphoinositide-3 kinase pathway; and the PKC ε pathway leading to activation of mitogen activated protein kinase. These pathways were shown to mediate the effect of carbachol on neurite outgrowth as well as the increased expression of fibronectin, further substantiating the important role of the latter in astrocyte-mediated neuritogenesis. Interference with these signaling pathways would be expected to impair astrocyte–neurons communication leading to impaired neuronal development.

1. Introduction

Neurite outgrowth is a fundamental event in brain development, as well as in regeneration of damaged nervous tissue (Kiryushko et al., 2004). Astrocytes express and/or release factors, such as fibronectin or laminin, which can promote neurite outgrowth (Costa et al., 2002; Tom et al., 2004). Limited information exist on the stimuli that may cause astrocytes to release neurite promoting factors, two of which have been reported to be thyroid hormone (T3) and vasoactive intestinal peptide (Martinez and Gomes, 2002; Trentin et al., 2003; Blondel et al., 2000).

Astrocytes have been shown to express cholinergic muscarinic receptors (Hosli and Hosli, 1993; Guizzetti et al., 1996); the acetylcholine analog carbachol, by activating muscarinic M_3 receptors in cortical or hippocampal astrocytes, promotes neurite outgrowth in hippocampal neurons (Guizzetti et al., 2008). Indeed astrocytes, stimulated for 24 h with carbachol (which is then washed-out), significantly increase neuritogenesis in hippocampal neurons upon a further 24 h co-culture. The main effect is an increase in the length of the longest neurite, identified as the axon by Tau-1 staining, and an increase in minor neurite length. These effects were found to be mediated by the release by carbachol-stimulated astrocytes of various neuritogenic factors, such as the extracellular matrix proteins fibronectin and laminin. Experiments with function-blocking antibodies indicated a primary role for fibronectin in carbachol-induced, astrocyte-mediated neuritogenesis in hippocampal neurons (Guizzetti et al., 2008). By activating muscarinic M_3 receptors, carbachol was found to increase the synthesis, expression, and release of fibronectin in astrocytes (Guizzetti et al., 2008; Moore et al., 2009).

Muscarinic M_3 receptors have been shown to activate a variety of signal transduction pathways (Caulfield, 1993), and over the years, we have characterized most of such signaling pathways in astroglial cells (Fig. 1). One pathway involves the activation of phospholipase D (PLD), the enzyme which hydrolyzes phosphatidylcholine, thereby generating choline and phosphatidic acid (PA) (Guizzetti and Costa, 2000; Guizzetti et al., 2004). PA activates the atypical protein kinase C ζ (PKC ζ), which in turn phosphorylates p70S6 kinase and nuclear factor κB (NF-κB) (Guizzetti and Costa, 2000, 2002; Guizzetti et al., 2003, 2004). The p70S6 kinase can also be activated by the phosphatidylinositol-3-kinase (PI-3K) pathway, which is also stimulated by muscarinic M_3 receptors in astroglial cells (Guizzetti and Costa, 2001). Finally, muscarinic receptors activate phospholipase C, which hydrolyzes phosphatidylinositol bisphosphate to generate inositol 1,4,5-trisphosphate and diacylglycerol (DAG); the former

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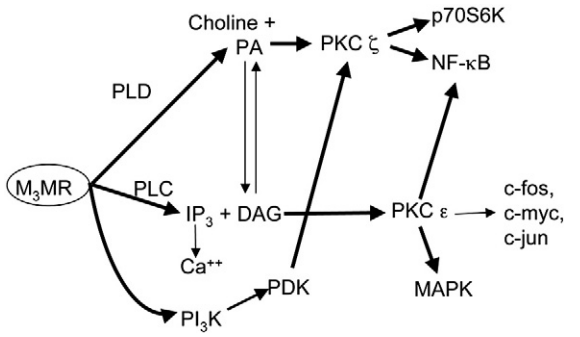

Fig. 1. Schematic representation of pathways activated by M_3 muscarinic receptors (M_3MR) in astroglial cells. Shown in bold are those found in the present study to be involved in the neuritogenic effect of carbachol-stimulated astrocytes on hippocampal neurons. PLC, phospholipase C; PLD, phospholipase D; PA, phosphatidic acid; DAG, diacylglycerol; IP_3, inositol 1,4,5-trisphosphate; PKC, protein kinase C; NF-κB, nuclear factor-κB; MAPK, mitogen activated protein kinase; PI-3K, phosphoinositide-3 kinase; and PDK, phosphoinositide-dependent kinase.

mobilizes calcium from intracellular stores, while the latter activates classical and novel PKCs (Caulfield, 1993). In astrocytes, muscarinic M_3 receptors cause an increase in intracellular calcium (Catlin et al., 2000), but do not appear to activate classical, calcium- and DAG-dependent PKCs such as PKC α (Guizzetti et al., 1998). In contrast, the novel, DAG-dependent PKC ε was activated by muscarinic M_3 receptors in astroglial cells, and this PKC in turn activates mitogen-activated protein kinase (MAPK) (Yagle et al., 2001).

The aim of the present study was to investigate the signal transduction pathway(s) involved in the neuritogenic action of carbachol in astrocytes, by assessing their effects on neurite outgrowth and fibronectin expression.

2. Material and methods

2.1. Materials

Alexa fluor-555 and Alexa fluor-594 secondary antibodies, Hoechst 33342, tissue culture medium, serum and B27 supplements were from Invitrogen (Carlsbad, CA). Tissue culture vessels were from Corning (Acton, MA), while glass coverslips were from Fisher Scientific (Federal Way, WA). The anti-βIII-tubulin antibody was from Chemicon International (Temecula, CA). Rapamycin, wortmannin, Gö6976 [12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5 H-indolo(2,3-a) pyrrolo(3,4-c)-carbazole], U0126 [1,4-diamino-2,3-dicyano-1,4-*bis*(2-aminophenylthio)butadiene], PD98059 (2′-amino-3′-metoxyflavone), LY294002 (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one), GF109203X (bisindolylmaleimide I), and Bay 11-7082 {(E)3-[(4-methylphenyl)sulfonyl]-2-propenenitrile} were purchased from Calbiochem/EMD Biosciences (San Diego, CA). The peptide SN50, its negative control SN50 mock (SN50M), and the myristoylated pseudosubstrates to PKC ζ and PKC α/β were purchased from Enzo Life Sciences (Plymouth Meeting, PA). All other chemicals and antibodies were from Sigma-Aldrich (St. Louis, MO). Time-pregnant Sprague–Dawley rats were purchased from Taconic Farms (Hudson, NY).

2.2. Primary cultures of hippocampal neurons

Hippocampal neurons from E21 rat fetuses were prepared as previously described in detail (Guizzetti et al., 2008; VanDeMark et al., 2009). Briefly, rat hippocampi were removed and dissected into 1 to 3 mm^3 pieces in Hank's balanced salt solution (HBSS), and treated with papain (2 mg/ml in HBSS) in the presence of DNase (40 μg/ml) and MgCl (5 mM) for 3 min at 37 °C. The tissue was spun down, and resuspended in Neurobasal medium supplemented with 10% fetal bovine serum (FBS), 30 mM glucose, 3 mM GlutaMAX, 1% gentamycin, 0.5% fungizone, and DNase (40 μg/ml). Tissue was further dissociated by repeated passages through a Pasteur pipette, and cells were filtered through a nylon mesh of 40 μm pore size. Cells were then spun down and resuspended in Neurobasal medium. For quantitative morphological analysis of neurite outgrowth, neurons (1×10^4 cells/coverslip) were plated in glass coverslips, coated overnight with 100 μg/ml poly-D-lysine to support neuron attachment, to which 3–4 beads of paraffin were previously affixed. After 30 min incubation in Neurobasal/B27 medium to allow for neurons to attach, the glass coverslips were inverted in 24-well plates above the astrocyte monolayer, the paraffin drops preventing direct neuron–astrocyte contact.

2.3. Primary culture of astrocytes

Primary cultures of cortical astrocytes from E21 rat fetuses were prepared as previously described in detail (Guizzetti et al., 1996, 2003, 2008). Briefly, rat cortices were dissected mechanically dissociated and incubated with trypsin, followed by trituration, repeated washing and filtering. After counting, cells were plated at a density of 10^7 cells/75 cm^2 tissue culture dish, pre-coated with poly-D-lysine, and grown in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5%CO_2/95%O_2. After nine days in culture, astrocytes were plated for experiments in 24-well plates for astrocyte–neuron co-culture experiments, or on glass coverslips for immunocytochemistry experiments (2.5×10^5 cells/well or coverslip); three-four days later, cells were serum-deprived for 24 h before treatments.

2.4. Treatments of astrocyte–neuron co-cultures

Astrocytes were treated with carbachol in the absence or presence of inhibitors of various signal transduction pathways (as indicated in Results) for 24 h. The treatments were then washed out, and neurons were added to the astrocyte monolayer for an additional 24 h.

2.5. Quantitative morphological analysis of neurite outgrowth

Morphological analysis has been previously described in detail (Guizzetti et al., 2008). Briefly, at the end of each co-incubation, neurons plated on coverslips were fixed in 4% formaldehyde, permeabilized in 0.2% Triton X-100, labeled with an anti-βIII-tubulin antibody followed by an Alexa fluor-555 secondary antibody, and mounted on microscope slides. Only stage 3 pyramidal neurons were selected for analysis. Stage 3 pyramidal neurons were those with three or more extensions, a cell body diameter of 10 to 15 μm, two to five undifferentiated neurites, and a single axon with length >40 μm (Dotti et al., 1988). Neurons whose processes were intermingled with those of neighboring cells were excluded from the analysis. Neurite length was measured from the point of emergence at the cell body to the tip of each segment. Images were obtained with a NIKON Labphot 2A microscope and projected to a Spot RT Slider cooled CCD digital camera. Quantification of the morphological parameters was carried out using MetaMorph 6.1 analysis software. At least 20 cells per treatment were analyzed in each experiment. As we had previously shown that the longest neurite, identified as the axon by the axonal marker Tau-1, was the most affected by carbachol-treated astrocytes (Guizzetti et al., 2008), axonal length was measured in the present study. All analyses were carried out in a blind fashion.

2.6. Astrocyte immunocytochemistry

At the end of the incubation, astrocytes plated on glass coverslips were fixed in 4% formaldehyde without permeabilization, for immunostaining of fibronectin, as previously described in detail (Guizzetti et al., 2008). After blocking nonspecific binding sites with 10% goat serum, astrocytes were incubated with an anti-fibronectin