Molecular and Cellular Neuroscience 78 (2017) 1–8

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Phosphoinositide 3-kinase γ ties chemoattractant- and adrenergic control of microglial motility

Nadine Schneble [a], Caroline Schmidt [a], Reinhard Bauer [a], Jörg P. Müller [a], Shamci Monajembashi [b], Reinhard Wetzker [a,*]

[a] Institute of Molecular Cell Biology, Center for Molecular Biomedicine (CMB), University Hospital of Jena, Hans –Knöll –Straße 2, 07745 Jena, Germany
[b] Leibnitz Institute for Age Research, Fritz Lipmann Institute (FLI), Beutenberg-Straße 11, 07745 Jena, Germany

ARTICLE INFO

Article history:
Received 10 June 2016
Revised 15 September 2016
Accepted 3 November 2016
Available online 5 November 2016

Keywords:
CNS
Immune system
Microglia
Chemotaxis
PI3Kγ

ABSTRACT

Microglial motility is tightly controlled by multitude of agonistic and antagonistic factors. Chemoattractants, released after infection or damage of the brain, provoke directed migration of microglia to the pathogenic incident. In contrast, noradrenaline and other stress hormones have been shown to suppress microglial movement. Here we asked for the signaling reactions involved in the positive and negative control of microglial motility. Using pharmacological and genetic approaches we identified the lipid kinase activity of phosphoinositide 3-kinase species γ (PI3Kγ) as an essential mediator of microglial migration provoked by the complement component C5a and other chemoattractants. Inhibition of PI3Kγ lipid kinase activity by protein kinase A was disclosed as mechanism causing suppression of microglial migration by noradrenaline. Together these data characterize PI3Kγ as a nodal point in the control of microglial motility.

1. Introduction

Microglial cells are key mediators of the innate immune response in the central nervous system (CNS) (Kettenmann et al., 2011). Activation of microglia caused by insults or infections of the CNS is accompanied by a series of cellular responses including secretion of cytokines, generation of reactive oxygen species and increase of motility and phagocytic activities (Lull and Block, 2010; Rock et al., 2004; Streit et al., 2005).

Directed migration to regions of infection or injury is one of the early responses of activated microglial cells. Microglia react quickly with process extension towards increasing levels of chemoattractants released by concerned cells (Orr et al., 2009). Stimulation of microglia motility can be induced by immune mediators like C5a (complement system component 5a) but also by damage signals like ATP (Haynes et al., 2006; Nolte et al., 1996). Opposing effects have been observed for the stress mediator noradrenaline, which was shown to suppress ATP induced motility of microglia (Gyoneva and Traynelis, 2013). Signaling mechanisms controlling both activation and inhibition of microglial motility are widely unknown.

Our present study is aimed to add to the understanding of signaling pathways of microglia motility control. Previous investigations revealed the phosphoinositide 3-kinase species PI3Kγ as a mediator of migration of neutrophils and macrophages (Ferguson et al., 2007; Hirsch et al., 2000; Stephens and Hawkins, 2013; Vanhaesebroeck et al., 2012). Thus we asked for the role of PI3Kγ in the control of the motility of macrophage cognate microglia. Our data reveal PI3Kγ as a mutual mediator of chemoattractant induced motility of microglia and the suppressive effect of noradrenaline.

2. Material and methods

2.1. Antibodies

Antibodies were obtained from Cell Signaling (Danvers, USA): Phospho-(Ser/Thr) PKA Substrate (#9621), pAkt Ser473 (#4058), panAkt (#4691). N-terminal p110γ antibody used for immunoprecipitation was provided by the laboratory of Emilio Hirsch (Torino, Italy). The monoclonal p110γ- antibody H1 was produced in our lab (Hirsch et al., 2000; Stephens et al., 1997; Stoyanov et al., 1995). Further primary antibodies were purchased from Echelon (Salt Lake City, USA): PIP_3 (Z-P345), Abcam (Cambridge, UK): Iba-1 and Sigma (St. Louis, USA): β-Actin (A2228). HRP-coupled secondary anti–mouse and anti–rabbit antibodies were obtained from KPL (Weden, Germany). Secondary

Abbreviations: Akt, protein kinase Akt/PKB; ATP, Adenosine triphosphate; C5a, complement system component 5a; C5aR, complement system component 5a receptor; cAMP, Cyclic adenosine monophosphate; DAPI, 4′,6-diamidino-2-phenylindole; HRP, horseradish peroxidase; PIP_3, phosphatidylinositol (3,4,5)-trisphosphate; shRNA, small hairpin RNA.

* Corresponding author at: Institute of Molecular Cell Biology, CMB-Center for Molecular Biomedicine, University Hospital, Hans-Knöll-Strasse 2, D-07745 Jena, Germany.
E-mail addresses: Nadine.Schneble@med.uni-jena.de (N. Schneble), Caroline.Schmidt@med.uni-jena.de (C. Schmidt), Reinhard.Bauer@med.uni-jena.de (R. Bauer), Joerg.Mueller2@med.uni-jena.de (J.P. Müller), shamci@fli-leibniz.de (S. Monajembashi), Reinhard.Wetzker@uni-jena.de (R. Wetzker).

http://dx.doi.org/10.1016/j.mcn.2016.11.002
1044-7431/

fluorescent donkey anti-goat isotype-specific antibody Alexa Fluor 568 and secondary fluorescent donkey anti-mouse isotype-specific antibody Alexa Fluor 350 were purchased from Invitrogen (Carlsbad, California, USA).

2.2. Inhibitors and other chemicals

Rolipram (10 μM) as a cAMP phosphodiesterase (PDE) 4 inhibitor, cilostamide (PDE 3 inhibitor, 10 μM) and IBMX (panPDE inhibitor, 500 nM) were obtained from Sigma (St. Louis, USA). H89 (10 μM) was purchased from Enzo Life Sciences (New York, USA). The adenylate cyclase activator forskolin (10 μM) was obtained from Enzo Life Sciences (New York, USA). M-CSF (30 ng/ml), NGF-β (30 ng/ml), MCP-1 (CCL2, 100 ng/ml) and SDF1-α (CXCL12, 20 nM) were purchased from ImmunoTools (Friesoythe, Germany). ATP (30 μM) and NECA (30 μM) were obtained from Sigma (St. Louis, USA) (Orr et al., 2009). Further chemoattractants were purchased from R&D systems (Minneapolis, USA): C5a, Biolegend (San Diego, USA): CX3CL1 and Abcam (Cambridge, UK): monomeric amyloid-β (1–42) peptide.

2.3. BV-2 cells

BV-2 an immortalized murine microglia cell line was cultured at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) high glucose from PAA Laboratories (Dartmouth, USA) containing 10% fetal calf serum (FCS), 1% penicillin/streptomycin and 1% amphotericin B.

2.4. shRNA-mediated down regulation of gene expression

The generation of specific shRNA cell lines has been described previously (Bekhite et al., 2011). Plasmid pLKO.1 vectors encoding shRNA constructs targeting PI3K p110α, β, γ or δ catalytic subunits and regulatory p84 and p101 subunits or plasmid pLKO.1 encoding a non-targeting control shRNA were obtained from the Sigma (St. Louis, USA). For generation of lentiviral particles, HEK293T cells were transfected with pLKO.1 derivative plasmids in combination with pRev, pEnv-VSV-G, and pMDLg. BV-2 cells were infected three times with the pseudotyped particles in the presence of 8 mg/ml polybrene. The transduced cell pools were selected with 2 mg/ml puromycin 48 h post transduction for 10 days. Downregulation of the catalytic and regulatory subunits of the PI3K class I was tested by Western blot analysis and real time PCR. Successful knockdown has been shown previously (Schmidt et al., 2013).

2.5. Mouse lines

PI3Kγ knockout and PI3Kγ knockin (kinase-dead, kinase inactive) mice were on the C57BL/6J background. C57BL/6J wild-type mice were used as controls (Patrucco et al., 2004).

2.6. Primary microglia cells

The microglia cells were isolated from neonatal mouse cerebral cortex as described (Giulian and Baker, 1985). The cells were co-cultivated with astrocytes for 14 days at 37 °C and 5% CO_2 in DMEM high glucose containing 10% FCS, 1% penicillin/streptomycin and 1% amphotericin B. After 14 days adherent microglia were separated from astrocytes by adding PBS/EDTA and careful shaking. After harvesting microglial cells were seeded in well plates.

2.7. Immunocytochemistry

Primary microglial cells were seeded onto 12 mm poly-L-lysine coated plates. After starvation for 24 h cells were treated with 10 ng/ml C5a for 5 min. Then, cells were fixed with 5% formaldehyde solution for 15 min at 37 °C. The cells were washed twice with PBS for removing fixative for 10 min at 25 °C. Primary antibodies against Iba-1 and PIP_3 were applied to the cells and incubated at 4 °C overnight. After three washes with PBS, fluorescent dye-labeled secondary antibodies were applied to the cells for 1 h at room temperature. The cells were mounted to the slides and observed with LSM 710 confocal microscope (Carl Zeiss, Jena, Germany).

2.8. SDS-PAGE and western blotting

For quantification of protein expression and phosphorylation cells were seeded into 6-well plates and incubated at 37 °C (5% CO_2). After becoming adherent, cells were incubated overnight in DMEM medium w/o FCS and treated for 24 h with agonists or inhibitors. Thereafter cells were suspended in RIPA lysis buffer composed of 50 mM Tris/HCl pH 8; 150 mM NaCl, 1% (v/v) NP-40, 0.5% (v/v) deoxycholate, 0.1% (w/v) SDS, 100 mg/ml Pefa-Block, 1 mg/ml pepstatin, 10 mM sodium vanadate and 1 mg/ml leupeptin. Referring to a 20-min centrifugation at 13,500*g*, 4 °C, supernatants were mixed with 5× protein sample buffer (5% SDS, 33% glycerol, 25% β-mercaptoethanol) and heated to 95 °C for 5 min. Protein samples were separated on 10% polyacrylamide-Gel, transferred to a polyvinylidenfluoride membrane and immunoblotted with indicated antibodies followed by enhanced chemiluminescence reaction.

2.9. Immunoprecipitation

For PI3Kγ immunoprecipitation, lysis was done in 1% Triton x-100, 120 mM NaCl and 50 mM TrisHCl (pH = 8.0). Lysis buffer was freshly supplemented with proteinase and phosphatase inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM Pefabloc, 1 mM sodium orthovanadate) and lysis was allowed on ice for 15 min before thorough vortexing and centrifugation. PI3Kγ immunoprecipitations were performed by incubating pre-cleared protein extracts with protein G-sepharose beads (GE Healthcare and Life Sciences, Freiburg, Germany) and anti-PI3Kγ antibodies at 4 °C for 2 h. Followed by centrifugation (1 min, 3000 rpm, 4 °C) and four washing steps with lysis buffer and washing buffer (0.1 M Tris-HCl pH = 7.4; 0.5 M LiCl). Finally 2× protein sample buffer was added to the beads with precipitated proteins and samples were heated up to 95 °C. High stability of PI3Kγ complexes with the activating Gβγ subunits of heterotrimeric G proteins has been described in recent studies (Shymanets et al., 2012; Stoyanov et al., 1995).

Adjusted aliquots of the cell extracts were subjected SDS-PAGE, followed by transfer to PVDF membranes (Millipore, Bedford, MA, USA) and probing with Phospho-(Ser/Thr) PKA Substrate antibody. Afterwards membranes were reprobed with PI3Kγ antibody.

2.10. Lipid kinase activity assay

Following preincubation with 30 μM norepinephrine for 20 min, 10 μM forskolin for 30 min or 500 nM IBMX for 1 h BV2 cells were stimulated with for 5 min 10 ng/ml C5a. After cell lysis, PI3Kγ was immunoprecipitated (as previously described) using N-terminal p110γ antibody for 2 h. Followed by centrifugation (1 min, 3000 rpm, 4 °C) and six washing steps with lysis buffer, washing buffer (0.1 M Tris-HCl pH = 7.4; 0.5 M LiCl) and lipid kinase buffer (20 mM HEPES pH 7.4; 5 mM $MgCl_2$) each two times. Afterwards corresponding samples were incubated in lipid kinase buffer. Beyond this 10 μg phosphatidylinositol (PI), 10 μg phosphatidylserine (PS), ATP and 5 μCi of [^{32}P]-ATP were added to the immunoprecipitated p110γ. Afterwards, samples were incubated for 10 min at 30 °C while slow shaking. The reaction was stopped by adding HCl. The phases were separated by adding chloroform/methanol (1:1) mixture. The organic phase was collected, dried and resuspended in chloroform/methanol (2:1) mixture. The suspension was drop wise transferred onto the TLC plates and resolved with