Neuroscience 236 (2013) 21–30

EFFECTS OF THE RAS HOMOLOG RHES ON AKT/PROTEIN KINASE B AND GLYCOGEN SYNTHASE KINASE 3 PHOSPHORYLATION IN STRIATUM

L. M. HARRISON,[a]* S. H. MULLER[a] AND D. SPANO[b]

[a] *Neuroscience Center of Excellence, Louisiana State University Health Sciences Center, New Orleans, LA 70112, USA*

[b] *CEINGE Biotecnologie Avanzate and Dipartimento di Biochimica e Biotecnologie Mediche, Naples University, Italy*

Abstract—G protein-coupled receptors (GPCR) signal not only through heterotrimeric G proteins, but also through alternate pathways. Thus, dopamine D2 receptors in the striatum signal through $G_{\alpha}i/o$ and also by promoting formation of a multi-protein complex containing β-arrestin2, protein phosphatase 2A (PP2A), and Akt in order to dephosphorylate Akt. Lithium, on the other hand, disrupts this complex to increase Akt phosphorylation. Rhes is a striatally enriched GTP-binding protein that has been shown to inhibit dopamine receptor-mediated behavior and signaling through heterotrimeric G proteins. Therefore, our objective was to test whether Rhes similarly affects signaling through the Akt/GSK3 pathway in the striatum. Rhes$^{-/-}$ mice showed basally increased Akt and GSK3β phosphorylation relative to rhes$^{+/+}$ mice that was not further enhanced by lithium treatment. Furthermore, they responded to the D1/D2 agonist apomorphine with increased Akt and GSK3 phosphorylation. Co-immunoprecipitation experiments revealed that apomorphine treatment recruits PP 2A-C to Akt in both rhes$^{+/+}$ and rhes$^{-/-}$ mice. Lithium did not disrupt their interaction in rhes$^{-/-}$ mice as there was little basal interaction. Rhes co-immunoprecipitated with β-arrestins, suggesting that it is integral to the multi-protein complex. Thus, Rhes is necessary for Akt dephosphorylation by the striatal multi-protein complex, and in its absence, a lithium-treated phenotype results.

Key words: dopamine, lithium, G protein, striatum, Rasd2, β-arrestins.

INTRODUCTION

Rhes (Ras Homolog Enriched in Striatum) is an intermediate size GTP-binding protein with preferential expression in the striatum (Usui et al.,1994; Falk et al., 1999; Harrison and LaHoste, 2006; Harrison et al., 2008). It has multiple effects in striatal cells and has recently been shown to act as an E3 ligase for sumoylation (Subramaniam et al., 2009, 2010). In addition, Rhes has been shown to inhibit signaling by G protein-coupled receptors (GPCRs). For example, reporter gene activation by agonist-occupied β-adrenergic and thyroid-stimulating hormone receptors is inhibited by Rhes (Vargiu et al., 2004), as well as cAMP accumulation by dopamine receptor agonists (Harrison and He, 2011; Harrison, 2012). In vivo, rhes$^{-/-}$ mice show up-regulated protein kinase A (PKA) signaling as evidenced by increased phosphorylation of GluR1 at the PKA site (Ser845) (Errico et al., 2008). These findings point to a role for Rhes in signaling through traditional pathways of GPCRs—heterotrimeric G proteins, cAMP, and adenylyl cyclase (AC). However, as alternate pathways for GPCR signaling have now been defined, the question arises as to whether Rhes affects this signaling as well.

Although the family of GPCRs was so named because of members' ability to activate heterotrimeric G proteins in order to transmit signals into the cell (Hepler and Gilman, 1992; Oldham and Hamm, 2008), alternate signaling pathways are being increasingly identified. For example, β-arrestins, originally described as contributing to the termination of GPCR-mediated signals, are now known to scaffold alternate signaling pathways upon termination of heterotrimeric G protein signaling. Phosphorylation of receptors by G protein-coupled receptor kinases, followed by binding of β-arrestins, ''uncouples'' the receptor from the G protein, a process that allows for internalization of the receptor for recycling or degradation (Krupnick and Benovic, 1998; Perry and Lefkowitz, 2002). However, this uncoupling from the G protein pathway can actually allow the receptor to signal by alternate pathways. The role of β-arrestins has been expanded to include scaffolding of multi-protein complexes. For example, they can scaffold ERK kinases to allow signaling through this pathway independent of the G protein pathway (Wei et al., 2003; Shenoy et al., 2006). Thus, the family of G protein-coupled receptors may more accurately be termed ''7 transmembrane receptors'' (TMRs) to denote that post-receptor signaling can occur through more than one major pathway (Shenoy and Lefkowitz, 2011; Shukla et al., 2011).

*Corresponding author. Address: Neuroscience Center of Excellence, Louisiana State University Health Sciences Center, 2020 Gravier Street, New Orleans, LA 70112, USA. Tel: +1-504-599-0912; fax: +1-504-568-5801.

E-mail address: lhar14@lsuhsc.edu (L. M. Harrison).

Abbreviations: GPCR, G protein-coupled receptor; GSK3, glycogen synthase kinase-3; LiCl, lithium chloride; PKA, protein kinase A; TMR, transmembrane receptors; PP2A, protein phosphatase 2A; Rhes, Ras Homolog Enriched in Striatum.

0306-4522/12 $36.00
http://dx.doi.org/10.1016/j.neuroscience.2012.12.062

A recently defined β-arrestin scaffolded pathway of 7 TMRs involves signaling by Akt (protein kinase B). Upon activation of dopamine receptors in the striatum, a multi-protein complex forms, consisting of at least β-arrestin2, Akt, and protein phosphatase 2A (PP2A). This complex allows PP2A to dephosphorylate Akt at the threonine 308 residue, thus decreasing activation of the kinase. This decreased Akt activation, in turn, results in less phosphorylation of the downstream target glycogen synthase kinase-3 (GSK3) (Beaulieu et al., 2005). As GSK3 is constitutively active and is inhibited by phosphorylation at an N-terminal serine (Ser21 in GSK3α and Ser9 in GSK3β), this decreased phosphorylation activates the kinase (Sutherland et al., 1993; Cross et al., 1995). Although it is not yet known whether other striatal receptors can promote formation of this complex, or whether it is formed in other areas of the brain, it has been demonstrated that among dopamine receptors, D2 receptors, but not D1, promote its formation. D3 receptors contribute to complex formation, but are not necessary for it (Beaulieu et al., 2007).

GSK3, originally described for its ability as one of the kinases that phosphorylate glycogen synthase (Embi et al., 1980; Woodgett, 1990), is now appreciated to play major roles in neuronal function, including neurogenesis, synapse formation, and neurite outgrowth (Cole, 2012). Also, several neuropsychiatric disorders are postulated to involve perturbations in Akt/GSK3 signaling. For example, schizophrenia is associated with decreased Akt activity, likely from increased activity of dopamine D2 receptors, which would be postulated to increase GSK3 activity (Emamian et al., 2004; Blasi et al., 2011). The anti-manic drug lithium also affects Akt/GSK3 signaling. It has been known for many years that lithium can directly inhibit GSK3 (Klein and Melton, 1996; Stambolic et al., 1996). Recently, it has been shown that lithium can also inhibit GKS3 indirectly by disrupting the striatal Akt-containing multi-protein complex that favors GSK3 activation (Beaulieu et al., 2004, 2008). Studies with GSK3β$^{+/-}$ mice have shown that this kinase is an important locus for the behavioral effects of lithium as these mice show a lithium-treated phenotype in forced swim and locomotor tests (Beaulieu et al., 2004; O'Brien et al., 2004). Due to the importance of Akt/GSK3 signaling in neuropsychiatric disorders and the presence of a protein complex regulating this signaling in the striatum, we tested whether Rhes is involved in the regulation of striatal Akt and GSK3.

EXPERIMENTAL PROCEDURES

Materials

Lithium chloride (LiCl) and R-(−)-apomorphine hydrochloride hemihydrate were purchased from Sigma (St. Louis, MO). The following antibodies were purchased from Cell Signaling Technology (Danvers, MA): panAkt, phospho-Akt Thr308, phospho-Akt Ser473, GSK3α/β, phospho-GSK3α/β, PP2A-C, beta-arrestin 1/2, GAPDH. An additional anti-β-arrestin1/2 antibody from Santa Cruz (Santa Cruz, CA) was also used. The anti-Rhes antibody was from FabGennix (Frisco, Texas).

Animals

Rhes$^{+/+}$ and rhes$^{-/-}$ mice were generated as described previously (Spano et al., 2004) and backcrossed for 10 generations onto the C57BL/6 background. Male and female mice, aged 2–4 months, from heterozygous and homozygous matings were used for all experiments. Genotypes were verified by using PCR of tail biopsies. Mice were group-housed in a climate controlled vivarium placed on a 12-h light/dark cycle (lights on at 0600) with food and water provided ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee of the Louisiana State University Health Sciences Center and were in accord with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The ARRIVE guidelines were used in the design and execution of the animal experiments, and every effort was made to minimize the suffering of the animals and to reduce the number of animals used. For LiCl experiments, mice were injected intraperitoneally (IP) with 200 mg/kg LiCl or vehicle (sterile saline) and sacrificed by rapid decapitation 30 min later. Apomorphine (3 mg/kg) or its vehicle (0.1% ascorbate) was injected subcutaneously (SC), and mice were sacrificed 1 h later by rapid decapitation. All injections were in a volume of 10 ml/kg.

Western blotting for phosphoproteins

For analysis of phosphoproteins, striata were rapidly dissected and homogenized in a buffer containing 50 mM Tris (pH = 8), 150 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, Sigma protease inhibitor cocktail, 0.3 μM okadaic acid, and 2 mM sodium orthovanadate. Protein concentration was determined with a Bio-Rad (Hercules, CA) Detergent Compatible Protein Assay kit. Proteins (20-μg cell lysate) were separated by SDS–PAGE using any kD polyacrylamide TGX gels (Bio-Rad) and transferred to PVDF membranes. After blocking with 5% Carnation® milk in TBS-T (20 mM Tris, 150 mM NaCl, 0.1% Tween-20), membranes were incubated at 4 °C overnight in TBS-T/5% BSA with primary antibody [pAkt308, pAkt473, Akt, pGSK3α/β, GSK3α/β (all 1:1000), or GAPDH (1:5000)]. Membranes were washed and incubated in HRP-conjugated secondary antibody (1:20,000; Pierce/Thermo Scientific; Rockford, Ill), followed by additional washing. Proteins were visualized by enhanced chemiluminescence (Pierce/Thermo) and imaged with a GE LAS4010 (GE Healthcare; Pittsburgh, PA). Blots were initially probed for phosphoproteins, then stripped (One Minute stripping buffer, GM Biosciences, Inc; Frederick, MD) and re-probed for respective total protein. GAPDH was used as a loading control.

Immunoprecipitations

Mice were injected as described above with LiCl, apomorphine, or their respective vehicles and sacrificed either 30 min (LiCl) or 1 h (apomorphine) later. Brains were rapidly dissected out, immediately frozen in isopentane at −50 °C, and stored at −80 °C until processing. Striata were rapidly dissected on ice by cutting a section containing anterior and middle striatum, and taking a 2-mm punch from each hemisphere. Striata were homogenized in a buffer containing 20 mM Tris, pH = 8, 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, 2 mM sodium orthovanadate, 2 μM okadaic acid, and Sigma protease inhibitor cocktail. Protein was determined as above. Immunoprecipitations were performed with the Catch and Release system (Millipore; Billerica, MA). Lysates (500 μg) were incubated with anti-Akt or anti-β-arrestin1/2 antibody (1:100) for 30 min at room temperature. In some apomorphine experiments, lysates were also incubated for 30 min at room