Cellular Signalling xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Cellular Signalling

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

5-HT1A receptor-mediated phosphorylation of extracellular signal-regulated kinases (ERK1/2) is modulated by regulator of G protein signaling protein 19☆

Q1 Qin Wang [a], Akiko Terauchi [b,1], Christopher H. Yee [b], Hisashi Umemori [b,1], John R. Traynor [a,*]

[a] Department of Pharmacology, University of Michigan, Ann Arbor, MI, USA
[b] Molecular & Behavioral Neuroscience Institute, University of Michigan, Ann Arbor, MI, USA

ARTICLE INFO

Article history:
Received 4 April 2014
Accepted 27 April 2014
Available online xxxx

Keywords:
Regulator of G protein signaling
Serotonin 1A receptor (5-HT1A)
FGF2
MAP kinase
SH-SY5Y cells
Hippocampal neurons

ABSTRACT

Q2 The 5-HT1A receptor is a G protein coupled receptor (GPCR) that activates G proteins of the Gαi/o family. 5-HT1A receptors expressed in the raphe, hippocampus and prefrontal cortex are implicated in the control of mood and are targets for anti-depressant drugs. Regulators of G protein signaling proteins (RGSs) are members of a large family that play important roles in signal transduction downstream of G protein coupled receptors (GPCRs). The main role of RGS proteins is to act as GTPase accelerating proteins (GAPs) to dampen or negatively regulate GPCR-mediated signaling. We have shown that a mouse expressing Gαi2 that is insensitive to all RGS protein GAP activity has an anti-depressant-like phenotype due to increased signaling of postsynaptic 5-HT1A receptors, thus implicating the 5-HT1A receptor–Gαi2 complex as an important target. Here we confirm that RGS proteins act as GAPs to regulate signaling to adenylate cyclase and the mitogen-activated protein kinase (MAPK) pathway downstream of the 5-HT1A receptor, using RGS-insensitive Gαi2 protein expressed in C6 cells. We go on to use short hairpin RNA (shRNA) to show that RGS19 is responsible for the GAP activity in C6 cells and also that RGS19 acts as a GAP for 5-HT1A receptor signaling in human neuroblastoma SH-SY5Y cells and primary hippocampal neurons. In addition, in both cell types the synergy between 5-HT1A receptor and the fibroblast growth factor receptor 1 in stimulating the MAPK pathway is enhanced following shRNA reduction of RGS19 expression. Thus RGS19 may be a viable new target for anti-depressant medications.

1. Introduction

Regulators of G protein signaling (RGS) proteins make up a large family of proteins comprising over 20 members that are defined by the presence of a RGS homology (RH) domain. This region of the protein binds to the active form of Gαi/o and Gαq proteins to accelerate GTP hydrolysis and form inactive Gα-GDP. Thus, RGS proteins act as GTPase accelerating proteins (GAPs) and afford a negative modulation of G-protein coupled receptor (GPCR) signaling [1–3]. Mutation of a glycine to serine in the switch I region of Gαi/o and Gαq proteins prevents the binding of Gα subunits to RH domain of all RGS proteins and so inhibits this negative regulation, leading to increased downstream signaling [4–8].

We have shown that a mouse with knock-in of a mutant Gαi2 that does not bind to RH domains (Gαi2GSGS) exhibits an anti-depressant- and anti-anxiety-like phenotype [9]. The phenotypic behavior is reversed by the 5-HT1A receptor antagonist WAY-100635. Moreover, heterozygotes show enhanced responses to the 5-HT1A receptor agonist 8-OH-DPAT and the selective serotonin reuptake inhibitor (SSRI) fluvoxamine in tests for anti-depressant-like activity. This behavior is hypothesized to be due to an enhanced activity of postsynaptic 5-HT1ARs in the hippocampus and/or prefrontal cortex,

Abbreviations: GPCRs, G protein coupled receptors; RGS, regulator of G protein signaling; GAP, GTPase accelerating protein; RH, RGS homology domain; shRNA, short hairpin RNA; GFP, green fluorescent protein; IBMX, 3-isobutyl-1-methylxanthine; MAPK, mitogen-activated protein kinase; AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; 5-HT1AR, serotonin receptor 5-HT1A; FGFR1, fibroblast growth factor receptor 1; FGF2, fibroblast growth factor 2 for FGFR1; GIPN, GAIP-interacting protein N-terminus; GIPC, GAIP-interacting protein C-terminus; PTX, pertussis toxin; 8-OH-DPAT, (±)-8-hydroxy-2-dipropylaminotetralin hydrobromide; GDP, guanosine diphosphate; GTP, guanosine triphosphate; RIPA, radio-immunoprecipitation assay; ANOVA, analysis of variance; i.c.v., intracerebroventricular injection; WAY-100635, *N*-[2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridyl)cyclohexanecarboxamide; SSRIs, selective serotonin re-uptake inhibitors or serotonin-specific reuptake inhibitor; GSK3β, glycogen synthase kinase 3 beta; Trk, tyrosine kinase.

☆ This work was supported by the National Institutes of Health [Grants: MH083754 and MH097021].

* Corresponding author at: Department of Pharmacology, University of Michigan, 1301 MSRB III, 1150 West Medical Center Drive, Ann Arbor, MI 48109-5632, USA. Fax: +1 734 763 4450.
E-mail address: jtraynor@umich.edu (J.R. Traynor).
[1] Current address: Department of Neurology, F.M. Kirby Neurobiology Center, Boston Children's Hospital, Harvard Medical School, Boston, MA 02115, USA.

http://dx.doi.org/10.1016/j.cellsig.2014.04.017
0898-6568/

rather than autoreceptors in the raphe nucleus, and is specific for Gαi2 [9]. However, the particular RGS protein or proteins that are responsible for endogenous GAP activity at these postsynaptic 5-HT1A receptors and whose action is inhibited by the genetic manipulation of Gαi2 have not been identified. Very recently, Stewart et al. [10] have shown that a RGS6 knock-out mouse exhibits very similar anti-depressant- and anti-anxiety-like phenotypic behaviors to the Gαi2GSGS mouse. On the other hand the underlying biochemistry appears to be different between the genotypes, with the Gαi2GSGS mouse showing alteration compared to wild-type littermates in the phosphorylation status of GSK3β [9], while the RGS6 mouse does not show these changes but rather variation in the level of phosphor-CREB [10]. Therefore, we have sought alternative candidate RGS proteins that could be responsible and/or contribute to the phenotypic 5-HT1A receptor-mediated anti-depressant-like behavior in the Gαi2GSGS mouse.

There is evidence that in addition to selectivity for Gα subtypes RGS proteins show specificity for particular receptors [11–14]. Studies of the effect of RGS proteins on 5-HT1A receptor signaling are limited. In addition to the studies with the RGS6 knockout mouse, RGS4, RGS10 and RGS20 have been reported to significantly attenuate 5-HT1A receptor signaling using heterologous cell lines and/or overexpression or inhibition of RGS proteins in raphe or cortical neurons [11,15,16]. We have previously demonstrated that RGS19, also known as Gα interacting protein or GAIP [17], acts as an effective GAP for mu-opioid receptor signaling in SH-SY5Y cells [14]. These cells also endogenously express 5-HT1A receptors [18]. 5-HT1A receptors and mu-opioid receptors show cross-talk [18] and also form functional heterodimers [19]. Therefore we hypothesized that RGS19 would act as an effective GAP for 5-HT1A receptor signaling.

Here we use C6 cells expressing RGS-insensitive Gαi2 to confirm endogenous GAP activity at 5-HT1A receptors linked to Gαi2. We then employ lentiviral delivery of short hairpin RNA (shRNA) to demonstrate that endogenous RGS19 protein in C6 cells, SH-SY5Y cells as well as mouse primary hippocampal neurons acts as a GAP for 5-HT1A receptor signaling in a brain region that is involved in the actions of serotonergic drugs in mood disorders. Finally, since fibroblast growth factor-2 (FGF2) acting at the FGF receptor 1 is reported to synergize with 5-HT1A receptor activation in hippocampal neurons [20], we ask if this synergy is modulated by RGS19.

2. Materials and methods

2.1. Cell culture

C6 and SH-SY5Y cells from ATCC are maintained in Dulbecco's modified Eagles medium (DMEM) with high glucose, l-glutamine, and pyridoxine HCl, without sodium pyruvate containing 10% fetal bovine serum (FBS, Invitrogen) under 5% CO_2 at 37 °C. C6 cell line stably expressing either pertussis toxin (PTX) insensitive Gαi2 (Gαi2CI) or both PTX insensitive and RGS insensitive Gαi2 (Gαi2CIGS) has been previously described [8,21].

Preparation of primary culture of hippocampal neurons is as follows: dissociated hippocampal cell culture was prepared as described previously [22]. Briefly, hippocampi were removed from postnatal day 0 (P0) mice (ICR) and dissociated with 0.5% trypsin. Dissociated hippocampal cells were plated at 60,000 cells per coverslip on 12 mm glass coverslips coated with poly-D-lysine (Millipore) in 24 well-plates, and maintained in neurobasal medium with B27 supplement (Invitrogen). All animal care and use were in accordance with the institutional guidelines and approved by the University Committee on Use and Care of Animals.

2.2. Western blot analysis

Whole-cell lysates were prepared from SH-SY5Y cells, C6 cells, and primary culture of hippocampal neurons as previously described [13]. Briefly, cells were suspended in ice-cold radio-immunoprecipitation assay (RIPA) lysis buffer containing protease inhibitor cocktail [13], then homogenized, and centrifuged at 20,000 ×*g* for 10 min. The supernatant (~20 μg) was subjected to SDS-PAGE on a 12% mini-gel and transferred to an Immobilon-P transfer membrane. The membrane was blocked with 1% bovine serum albumin in TBST (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 1 h and incubated with RGS19-specific or RGS4-specific anti-serum at a 1:8000 dilution overnight on a rocking shaker in the cold room. After three consecutive washes, the membrane was incubated with 1:10,000 dilution of secondary antibody (goat anti-rabbit IgG-horseradish peroxidase) for 1 h at room temperature. Prestained SDS-PAGE protein standards (Bio-Rad, Precision Plus Protein standards, Kaleidoscope) were used to determine the size of detected proteins. The membranes were cut at the 37-kDa marker, and the upper membrane was blotted with anti-α-tubulin antibody at 1:10,000 dilution as an internal control for protein loading. Proteins were visualized by chemiluminescence with SuperSignal West Pico (Pierce) and exposed to X-ray film or using the Odyssey FC imaging system (LI-COR, Inc., Lincoln, NE), then quantified.

2.3. cAMP assay

One day before transfection, stable PTX insensitive (PTX-i) and RGS/PTX insensitive (RGS/PTX-i) Gαi2 expressing C6 cells were plated into a 24-well plate so as to reach 60–80% confluency at transfection. HA tagged 5-HT1A plasmid DNA was transiently transfected with Lipofectamine 2000 for 48 h. Cells were treated with PTX (100 ng/ml) overnight before assay. On the day of assay, cells were washed once with fresh serum-free medium, and the medium was replaced with 1 mM 3-isobutyl-1-methylxanthine (IBMX) in serum-free medium for 15 min at 37 °C and then replaced with medium containing 1 mM IBMX, 30 μM forskolin, and 10 μM 8-OH-DPAT for 5 min at 37 °C. Reactions were stopped by replacing the medium with ice-cold 3% perchloric acid, and samples were kept at 4 °C for at least 30 min. An aliquot (0.4 ml) from each sample was removed, neutralized with 0.08 ml of 2.5 M $KHCO_3$, vortexed, and centrifuged at 15,000 ×*g* for 1 min to pellet the precipitates. Accumulated cAMP was measured by radioimmunoassay in a 15 μl aliquot of the supernatant from each sample following the manufacturer's instructions (Diagnostic Products, Los Angeles, CA) and calculated as pmol/μg protein accumulation of cAMP.

2.4. MAPK assay

Cells were plated in 24-well plates and treated with PTX (100 ng/ml) for 7–8 h in serum-free medium for C6 cells before assay. For SH-SY5Y cells, they were in serum-free medium for 48 h. For primary culture of hippocampal neurons, they were in serum-free medium for 3 h only. Then cells were washed once with fresh serum-free medium and stimulated with varying concentrations of 8-OH-DPAT (Sigma-Aldrich) as indicated with or without FGF2 (0.5 ng/ml, Sigma-Aldrich) or distilled H_2O, respectively, for 5 or 10 min at 37 °C. The reaction was stopped by adding 0.1 ml of ice-cold SDS sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.01% bromophenol blue), and samples were removed from the wells, boiled for 5 min, and then subjected (10–15 μl each) to electrophoresis using a 12% SDS-PAGE mini-gel, followed by transfer to an Immobilon™-FL membrane (Fisher) for Western blotting. After blocking with an Odyssey blocking buffer for 1 h, the blot was probed with a 1:1000 dilution of mouse anti-phospho-p44/42MAPK (Thr202/Tyr204) antibody and rabbit anti-p44/42 MAPK antibody (Cell Signaling Technology) overnight on a rocking shaker in a cold room. After three consecutive washes, the blot was incubated with secondary antibody (1:10,000 dilution) of anti-mouse IRdye 680 RD and anti-rabbit IRdye 800 CW for 1 h at room temperature. Then images were acquired using an Odyssey FC imaging system (Li-COR Biosciences, Lincoln, NE) and quantified using building in program. MAPK activity was calculated as the ratio of normalized

Please cite this article as: Q. Wang, et al., 5-HT1A receptor-mediated phosphorylation of extracellular signal-regulated kinases (ERK1/2) is modulated by regulator of G protein signaling protein 19, Cellular Signalling (2014), http://dx.doi.org/10.1016/j.cellsig.2014.04.017