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Neuropeptides 39 (2005) 547–558
Neuropeptides
www.elsevier.com/locate/npep

Multiple interaction sites of galnon trigger its biological effects

Anders Florén [a], Ulla Sollenberg [a], Linda Lundström [a], Matjaž Zorko [b], Jure Stojan [b], Metka Budihna [c], Mark Wheatley [d], Negin P. Martin [e], Kalle Kilk [f], Andrey Mazarati [g], Tamas Bartfai [h], Maria Lindgren [a], Ülo Langel [a,*]

[a] *Department of Neurochemistry, Stockholm University, S. Arrheniusv 21 A, SE-106 91 Stockholm, Sweden*
[b] *Institute of Biochemistry, Medical Faculty, University of Ljubljana, Vrazov trg 2, 1000 Ljubljana, Slovenia*
[c] *Institute of Pharmacology and Experimental Toxicology, Medical Faculty, University of Ljubljana, Korytkova 2, 1000 Ljubljana, Slovenia*
[d] *School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK*
[e] *The Howard Hughes Medical Institute, Duke University Medical Center, Box 3821, Durham, NC, 27710, USA*
[f] *Centre of Molecular and Clinical Medicine, Tartu University, Ravila 19, 51014 Tartu, Estonia*
[g] *Department of Neurology, University of California, Los Angeles, West Los Angeles Veteran Administration Medical Center, 90073, Los Angeles, CA, USA*
[h] *Department of Neuropharmacology, The Scripps Research Institute, La Jolla, CA 92037, USA*

Received 23 May 2005; accepted 24 September 2005
Available online 16 November 2005

Abstract

Galnon was first reported as a low molecular weight non-peptide agonist at galanin receptors [Saar et al. (2002) *Proc. Natl. Acad. Sci.* USA **99**, 7136–7141]. Following its systemic administration, this synthetic ligand affected a range of important physiological processes including appetite, seizures and pain. Physiological activity of galnon could not be explained solely by the activation of the three known galanin receptors, GalR1, GalR2 and GalR3. Consequently, it was possible that galnon generates its manifold effects by interacting with other signaling pathway components, in addition to via GalR1-3. In this report, we establish that galnon: (i) can penetrate across the plasma membrane of cells, (ii) can activate intracellular G-proteins directly independent of receptor activation thereby triggering downstream signaling, (iii) demonstrates selectivity for different G-proteins, and (iiii) is a ligand to other G-protein coupled receptors (GPCRs) in addition to via GalR1-3. We conclude that galnon has multiple sites of interaction within the GPCR signaling cascade which mediate its physiological effects.

Keywords: Galanin; Galnon; G-protein; Receptor; Signaling

Abbreviations: AR, adrenoreceptor; AVP, [Arg^8]vasopressin; CHO, Chinese hamster ovary; D1(2)R, dopamine receptors, type 1 (2); DMSO, dimethylsulfoxide; GalR, galanin receptor; GPCR, G-protein coupled receptors; HKR, Hepes–Krebs–Ringer solution; HPLC, high performance liquid chromatography; 5-HT, 5-hydroxytryptamine; IC_{50}, concentration giving half-maximal inhibition; LC–MSD-Trap-XCT, liquid chromatograph–mass spectrometer-ion trap instrument; LDH, lactate dehydrogenase; M1, (2,5) mAChR muscarinic acetylcholine receptor, type 1 (2, 5); mAChR, muscarinic acetylcholine receptor, not specified; MALDI-TOF MS, matrix assisted laser desorption/ionization-time-of-flight mass spectrometer; MC3 (4), melanocortin receptor, type 3 (4); OTR, oxytocin receptor; PBS, phosphate buffered saline; PTX, pertussis toxin; PPS, perforant path stimulation; RP, reverse phase; SSSE, self-sustaining status epilepticus; TFA, trifluoroacetic acid; V_{1a}R, V_{1a} vasopressin receptor; V_{1b}R, V_{1b} vasopressin receptor; V_2R, V_2 vasopressin receptor.

* Corresponding author. Tel.: +46 816 1793; fax: +46 816 1371.
E-mail address: ulo@neurochem.su.se (Ü. Langel).

0143-4179/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.npep.2005.09.005

1. Introduction

G-protein coupled receptors (GPCR) comprise one of the most important pharmaceutical targets, with 60% of all clinically prescribed drugs being GPCR ligands. Alternative therapeutic agents which bypass GPCR, but rather directly target downstream intracellular signaling cascades have not been developed to-date.

Galanin receptors (GalRs) are members of the rhodopsin-like family A GPCR superfamily of proteins. Three subtypes of GalRs have been identified, termed GalR1, GalR2 and GalR3. In an attempt to generate a novel ligand for these receptors, a combinatorial library was constructed based on a tripeptide which incorporated the major pharmacophore of the natural agonist galanin, namely Trp^2, Asn^5 and Tyr^9. Screening of this library identified galnon, 7-[(9-fluorenylmethoxycarbonyl) cyclohexylalanyllysyl] amino-4-methylcoumarin, a low molecular weight substance (MW of 677 Da) capable of displacing [^{125}I]-porcine galanin binding to the GalR1 with an affinity of 3–12 μM (Bartfai et al., 2004; Saar et al., 2002). Galnon was subsequently demonstrated to be an agonist at this receptor as it inhibited adenylyl cyclase in rat hippocampal membranes (Saar et al., 2002), which are known to be rich in GalR1.

Administration of galnon generates a wide range of physiological effects. For example, galnon has been reported to act as an anti-convulsant in vivo (Saar et al., 2002), to decrease symptoms of opiate withdrawal (Zachariou et al., 2003), to regulate appetite, thereby reducing food intake (Abramov et al., 2004), to induce long-term potentiation in dentate gyrus of C57BL/6 mice (Badie-Mahdavi et al., 2005), to increase insulin release in rat pancreatic islets (Quynh et al., 2005), to produce an antidepressant effect in rats (Lu et al., 2005a), and to alleviate nociceptive responses to partial sciatic nerve injury (Wu et al., 2003); summarized in (Sollenberg et al., 2005). While some of the effects of galnon have been shown to involve GalR, others cannot be explained solely by this mechanism. Hence, additional pathways to interaction of galnon with galanin receptors must be involved to explain the observed results. For example, intraperitoneally applied galnon prolongs heat withdrawal latency in sciatic nerve injured rats in a dose-dependent manner. However, galnon does not affect mechanical or cold hypersensitivity, even though this effect was observed previously with intrathecally applied galanin (Wu et al., 2003). Furthermore, while intracerebroventricular injection of galanin stimulates food consumption (Kyrkouli et al., 1990), galnon causes a pronounced, dose-dependent, reduction of food intake in rats and mice following both intraperitoneal and intracerebroventricular administration (Abramov et al., 2004). In vivo anticonvulsant activity of galnon was comparable to that of galanin despite the differences in the binding affinity between the two compounds (Saar et al., 2002). Taken together, these data suggest that galnon must have other modes of action in addition to the direct activation of GalRs.

It has been reported previously that a small number of compounds, such as neuropeptide Y and substance P, and several basic secretagogues, can activate G-proteins directly in a receptor-independent manner, albeit at micromolar concentrations (Ferry et al., 2002; Ogawa et al., 1999; Raimondi et al., 2002). In this study, we establish that galnon can cross the plasma membrane of cells to activate G-proteins directly and selectively. In addition, we demonstrate that galnon is also a ligand at GPCRs other than GalR1-3. The ability of a small molecule to affect G-proteins directly, rather than via a GPCR, may provide a novel approach for pharmaceutical intervention of intracellular signaling.

2. Materials and methods

2.1. Chemicals and reagents

Lipofectamine and Fugene were purchased from Life Technologies/Invitrogen (Carlsbad, CA) and Roche (Basel, Switzerland), respectively. SKF38393 was purchased from Research Biochemicals International (Natick, MA). [^{125}I]-(−) iodocyanopindolol, [*N*-Methyl-^{3}H]-SCH23390, [^{125}I]-galanin, [Phe^3-3,4,5-^{3}H]AVP, [*N*-methyl-^{3}H] scopolamine and [Tyr-2,6-^{3}H]oxytocin were all from Perkin–Elmer Life Sciences (Boston, MA). C18 ZipTips were obtained from Millipore (Billerica, MA). Amino acids were from Novabiochem (Darmstadt, Germany). Cellmedia and reagents were purchased from Life Technologies/Invitrogen (Carlsbad, CA) or Sigma–Aldrich (St. Louis, MO). AVP and OT were purchased from Bachem Ltd (St. Helens, UK). Soluene 350 (Packard, USA), HiSafe3^{TM} (Wallac, UK). Carboxyfluoroscein was purchased from Molecular Probes (Leiden, The Netherlands) and lactate dehydrogenase (LDH) leakage assay Cytotox-one, from Promega (SDSbiosciences, Sweden). Additional chemicals and reagents were all from SigmaAldrich (St. Louis, MO).

2.2. Cell cultures and transfection

HEK293 and chinese hamster ovary, CHO, cells were maintained in Minimal Essential Medium and F-12 Medium, respectively. COS-7 and HEK 293T were cultured in Dulbecco's Modified Eagle's Medium. Rinm5F cells were grown in RPMI-1640 medium with 2 mM L-glutamine. Bowes human melanoma cells (American Type Culture Collection CRL-9607) were grown in Eagle's Minimal Essential Medium with Glutamax with 1% non-essential amino acids and 1% sodium pyruvate. *Sf*9 cells were maintained in Grace's insect medium. All media were supplemented with 10% fetal bovine serum,