Biochemical Pharmacology 85 (2013) 1162–1170

Contents lists available at SciVerse ScienceDirect

Biochemical Pharmacology

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

Signaling of an allosteric, nanomolar potent, low molecular weight agonist for the follicle-stimulating hormone receptor

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ARTICLE INFO

Article history:
Received 17 December 2012
Accepted 4 February 2013
Available online 13 February 2013

Keywords:
FSH receptor
Ovary
Infertility
Allosterism
Agonist

ABSTRACT

Follicle-stimulating hormone (FSH) activates FSH receptors (FSHR) in granulosa cells to induce follicle differentiation, growth and estradiol production. FSH is used clinically to treat female infertility and is administered by injection. To increase patient convenience and compliance, compound homogeneity and composition, low molecular weight (LMW), orally bioavailable, FSHR agonists are now being developed to replace FSH. In this study, we present the signaling mechanisms of a newly developed LMW dihydropyridine agonist of the FSHR, Org 214444-0. Org 214444-0 is shown to be a stereoselective, nanomolar potent FSHR agonist and selective over the structurally related LHR and TSHR. Org 214444-0 is an allosteric agonist interacting with the transmembrane region of the FSHR. When co-incubated with FSH, Org 214444-0 augments FSH's potency in binding (6.5-fold) and adenylyl cyclase/cAMP activation (3.5-fold) in a concentration-dependent manner. Like FSH, Org 214444-0 induces FSHR internalization and is only marginally effective in stimulating phospholipase C. Moreover, Org 214444-0 stimulates cAMP and estradiol production in human granulosa cells in culture and supports the follicular phase in mature female rats. We conclude that Org 214444-0 is a bonafide FSHR agonist.

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1. Introduction

The pituitary gonadotropin FSH (follicle-stimulating hormone) and its receptor play a crucial role in reproductive function [1]. In females, FSH regulates follicle differentiation and maturation and stimulates estrogen production in granulosa cells. In males, FSH supports spermatogenesis by Sertoli cells. Deletion of the FSH receptor (FSHR) in female mice causes an arrest in follicular maturation at the preantral stage while smaller testis size is observed in male mice lacking FSHR [2,3]. In humans, patients with inactivating mutant FSHRs suffer from variable abnormalities of pubertal development with primary or secondary amenorrhea in females [3–10] and suppression of spermatogenesis in males [11]. Two common variants in the FSHR gene (Thr307Ala and Asn680Ser) have been identified, which show a modulating effect on FSH action in vivo [12].

The FSHR together with the luteinizing hormone receptor (LHR) and thyroid-stimulating hormone receptor (TSHR) constitute a highly conserved subgroup of G protein-coupled receptors which display the highest degree of amino acid conservation in the seven transmembrane domains (~70% amino acid identity) and a long, divergent extracellular amino terminus with ~40% sequence identity [13]. Activation of the FSHR and other glycoprotein hormone receptors requires the binding of the hormones to the *N*-terminus of the receptor and intramolecular signal transduction

Abbreviations: CRE, cyclic AMP-responsive element; E2, estradiol; FSH, follicle-stimulating hormone; FSHR, follicle-stimulating hormone receptor; GPCR, G protein-coupled receptor; G_s, stimulatory G protein; LH, luteinizing hormone; LHR, luteinizing hormone receptor; TSH, thyroid-stimulating hormone; TSHR, thyroid-stimulating hormone receptor.

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0006-2952/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bcp.2013.02.001

from the FSH–FSHR complex to the transmembrane domains of the receptor [14]. However, the mechanism that underlies this intramolecular signaling pathway is still far from understood, despite crystallographic studies pointing to FSH binding to the extracellular domain of the FSHR [15,16].

The main signaling pathway of FSHR in granulosa cells is stimulation of adenylyl cyclase at physiological concentrations of FSH (10–50 pM, corresponding to 5–20 U/l) via coupling to Gs proteins. This pathway is responsible for granulosa cell differentiation and growth, aromatase expression and LHR induction [17,18]. Stimulation of the FSHR also leads to activation of the ERK MAP kinase pathway in a cAMP-dependent manner [19]. In addition, supra-physiological concentrations of FSH may activate phospholipase C in granulosa and heterologously transfected cells [8,20,21]. Other but less characterized intracellular signaling pathways have been reported as well but whether they are relevant in vivo remains to be determined [22].

FSH serves a crucial role in assisted reproductive therapy of anovulatory females. Although FSH is highly effective in a significant number of patients, there are several aspects of the current fertility treatment with FSH that justify further improvement. First, FSH is a high molecular weight protein that needs to be administered by parenteral injection. Second, FSH is prepared by recombinant technology, involving CHO cells cultured in the presence of human or animal serum, of which traces may be present after purification or prepared through isolation from the urine of post-menopausal women and may show individual differences in glycosylation and sialylation resulting in different pharmacokinetic behavior. Third, FSH needs to be stored at low temperature to prevent decomposition. For these reasons, research and development within pharmaceutical companies have been focused on developing LMW FSHR agonists that offer increased homogeneity and consistency, better compound stability compared to the protein hormones and, preferably, can be administered orally to improve patient convenience and compliance. Indeed, in the past few years, LMW FSHR agonists from several compound series have been identified and optimized with potencies ranging from 1 to ~200 nM in heterologous screening cell-lines and granulosa cells. These include biaryl diketopiperazines [23,24], thienopyrimidines [25], dihydropyridines [26], thiazolidinones [27–29] as well as others [see for a review: [30]]. On the basis of previous work, most LMW FSHR agonists (and antagonists) are thought to be allosteric compounds, presumably interacting with the transmembrane domains instead of the *N*-terminus of the receptor [29,31–33]. Allosteric receptor agonists, however, can induce signaling pathways that are different from those induced by the native, orthosteric ligand [34]. Our knowledge about the allosteric modulation of the FSHR by LMW FSHR ligands, however, is remarkably scarce and there have been no reports yet on the functional characteristics of LMW FSHR agonists in animal models of fertility. In the present study, we have investigated for the first time the mechanism of action of a nanomolar potent, prototypic LMW FSHR agonist of the dihydropyridine compound series, Org 214444-0 (Fig. 1A). In vivo, Org 214444-0 shows oral bioavailability and mimicks the action of FSH in a follicular phase rat fertility model.

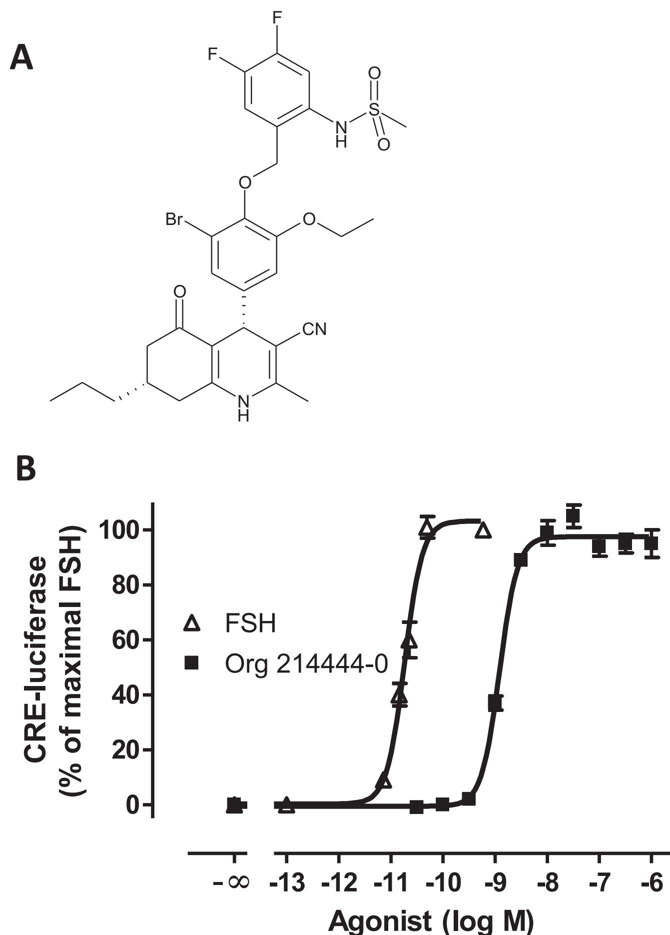

Fig. 1. Structural formula of Org 214444-0 (A). Effect of Org 214444-0 and FSH on CRE-luciferase activity in CHO cells stably expressing human FSHR together with CRE-luciferase reporter gene (B).
Data are from a single set of experiments (mean ± SD, done in quadruplicate), representative of 5 experiments. Corresponding EC_{50} values of Org 214444-0 and FSH are 1.2 nM and 18 pM, respectively. Control experiments showed that Org 214444-0 does not activate CRE-luciferase activity or cAMP synthesis in CHO cells expressing CRE-luciferase only (data not shown).

2. Materials and methods

2.1. Materials

Org 214444-0 (*N*-{2-[2-Bromo-4-((4R,7S)-3-cyano-2-methyl-5-oxo-7-propyl-1,4,5,6, 7,8-hexahydro-quinolin-4-yl)-6-ethoxy-phenoxymethyl]-4,5-difluoro-phenyl}-methane sulfonamide, MW 664.57) and its stereoisomer, Org 264935-0 were synthesized in house as described previously [26]. Two different batches of [^{125}I]recombinant human FSH were synthesized in house by radiodination of recombinant human FSH with Na [^{125}I]iodine (PerkinElmer, Groningen, The Netherlands) by the Iodogen method and purified by column chromatography to a specific activity of 1392 and 645 Ci/mmol. *Myo*-[2-^{3}H]inositol (spec. act. 21 Ci/mmol) was purchased from GE Healthcare Life Sciences, Diegem Belgium). Recombinant human FSH, recombinant human LH and recombinant human chorionic gonadotropin (hCG) were produced in house. TSH (bovine) was purchased from Sigma–Aldrich (Zwijndrecht, The Netherlands). Human LHR cDNA was received from Dr. Aaron J.W. Hsueh, Stanford University [35]), human TSHR cDNA from Dr. Edwin Milgrom, INSERM (Paris, France) [36]), rat FSHR cDNA from Dr Axel P.N. Themmen, Erasmus University Rotterdam (The Netherlands). The EGFP-fused rat FSHR-expressing U2OS celline was purchased from BioImage (now part of Thermo Fisher Scientific, Breda, The Netherlands). DME/F12 medium, Glutamax-1 DME medium, Hanks balanced salt solution (HBSS), custom-made inositol-free DME medium, Hoechst 33342, fetal calf serum, bovine calf serum, penicillin G, streptomycin sulfate, geneticin and hygromycin were purchased from Invitrogen (Breda, The Netherlands). Bovine insulin, human apo-transferrin, Hepes, dimetylsulfoxide (DMSO), rolipram, bovine serum albumin (BSA), 3-isobutyl-1-methylxanthine (IBMX),

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