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Persistent GnRH receptor activation in pituitary αT3-1 cells analyzed with a label-free technology

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ABSTRACT

The gonadotropin-releasing hormone (GnRH) receptor is a drug target for certain hormone-dependent diseases such as prostate cancer. In this study, we examined the activation profiles of the endogenous ligand, GnRH and a well-known marketed analog, buserelin using a label-free assay in pituitary αT3-1 cells with endogenous GnRH receptor expression. This whole cell impedance-based technology allows for the real-time measurement of morphological cellular changes. Both agonists dose-dependently decreased the impedance as a result of GnRH receptor activation with potencies of 9.3 ± 0.1 (pEC_{50} value, buserelin) and 7.8 ± 0.06 (pEC_{50} value, GnRH). Subsequently, GnRH receptor activation was completely abolished with a selective $G\alpha_q$ inhibitor, thereby confirming the $G\alpha_q$-coupling of the GnRH receptor in pituitary αT3-1 cells. Additionally, we observed continued responses after agonist stimulation of αT3-1 cells indicating long-lasting cellular effects. Wash-out experiments demonstrated that the long-lasting effects induced by GnRH were most likely caused by rebinding since over 70% of the original response was abolished after wash-out. In contrast, a long receptor residence time was responsible for the prolonged effects caused by buserelin, with over 70% of the original response remaining after wash-out.

In summary, we validated that impedance-based label-free technology is suited for studying receptor-mediated activation in cell lines endogenously expressing the target of interest. Moreover, this real-time monitoring allows the examination of binding kinetics and its influence on receptor activation at a cellular level.

1. Introduction

Label-free technologies can noninvasively monitor real-time receptor-mediated phenotypic responses in living cells encompassing all involved signaling pathways (Rocheville et al., 2013; Scott and Peters, 2010). Label-free whole cell assays typically use a biosensor to detect a ligand-induced cellular response by ways of acoustic, electrical or other quantifiable signals (Fang, 2011; Zhang and Xie, 2012). The main advantage of using biosensors and cell morphology as a readout is that cells can be assessed in their native and physiologically relevant environment bypassing the potentially negative effects of engineering on cell signaling (Xi et al., 2008; Yu et al., 2006). Additionally, label-free assays are highly sensitive therefore making them suitable for endogenous expression systems. Label-free studies are most commonly used to examine G protein-coupled receptor (GPCR) activation and G protein-signaling profiles (Deng et al., 2013; Schroder et al., 2010; Stallaert et al., 2012), but also for investigating cytotoxicity, cell adhesion, proliferation, migration and invasion (Atienza et al., 2006; Leurs et al., 2012; Xi et al., 2008).

The gonadotropin-releasing hormone receptor (GnRHR) is part of the rhodopsin family of GPCRs and is sub-classified in the β-group where all endogenous ligands are peptides (Fredriksson et al., 2003). Its endogenous ligand, gonadotropin-releasing hormone (GnRH) is a decapeptide synthesized in hypothalamic neurons. GnRH regulates the synthesis and secretion of luteinizing hormone (LH) and follicle stimulation hormone (FSH) by selectively stimulating pituitary gonadotropes expressing the GnRH receptor (Stojilkovic et al., 1994). The role of GnRHR in regulation of hormone levels in both males and females makes it an important target in hormone dependent diseases such as precocious puberty, fertility disorders and cancers of the prostate, mammary, ovary and endometrium (Labrie, 2014; McArdle, 2012).

In the current study we investigated GnRHR-induced signaling in a heterologous CHOhGnRH-NFAT cell line as well as the αT3-1 cell line using a label-free whole cell impedance-based assay. The gonadotrope mouse pituitary αT3-1 cell line (Windle et al., 1990) is known to have high endogenous GnRHR expression (Shah and Milligan, 1994). In the present study, we established that both the

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heterologous CHOhGnRH-FNAT cell line and the endogenous αT3-1 cell line are suitable to study GnRHR-mediated signaling using a label-free technology. In addition, we were able to, for the first time, elucidate the functional effects of GnRHR agonists with different binding kinetics. Taken together, we demonstrated the importance of monitoring integrated cellular responses to gain knowledge in receptor signaling and binding kinetics that cannot be detected with traditional endpoint assays.

2. Methods

2.1. Materials and reagents

GnRH, buserelin and cetrorelix were purchased from Sigma-Aldrich Chemie B.V. (Zwijndrecht, the Netherlands), while UBO-QIC was purchased from the Institute of Pharmaceutical Biology (University of Bonn, Germany). [2-^{3}H(N)]-myoinositol (specific activity 10–25 Ci/mmol), isoplate-96™ white frame, clear well plates and YSi Poly-L-Lysine coated SPA beads were purchased from Perkin Elmer (Boston, MA). CHO cells stably expressing both the human GnRH receptor and an NFAT reporter gene (CHOhGnRH-NFAT) were obtained from Invitrogen (Carlsbad, CA). αT3-1 cells were a kind gift from Dr. Pamela L. Mellon (Salk Institute, San Diego, CA). xCELLigence E-plate 16 and 96 were obtained from Westburg (Leusden, the Netherlands). All other compounds and materials were obtained from standard commercial sources.

2.2. Cell culture

CHOhGnRH-NFAT cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% dialyzed fetal calf serum (FCS), 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 100 μg/ml zeocin, 600 μg/ml hygromycin, 100 IU/ml penicillin and 100 mg/ml streptomycin at 37 °C+5% CO_2. αT3-1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum (FCS), 4.5 g/L glucose, 548 mg/L L-glutamine, 110 mg/L pyruvate, 100 IU/ml penicillin and 100 mg/ml streptomycin at 37 °C+5% CO_2. Cells were cultured as a monolayer and used for whole cell experiments when a confluency of ~75% was reached.

2.3. Label-free whole-cell assays

Label-free whole-cell assays were performed using the xCELLigence RTCA system (Xi et al., 2008; Yu et al., 2006) as described previously (Hillger et al., 2015).

CHOhGnRH-NFAT cells and αT3-1 cells were cultured as a monolayer on 10-cm ø culture plates and were harvested when confluency was around 75%. The experiment was started by adding 45 μl culture medium to each well to obtain background signal. Subsequently, 50 μl of cell suspension containing 1.6*10^6 cells/ml was added to each well to obtain approximately 40.000 cells/well. After roughly 18 h on the recording device station in a humidified atmosphere at 37 °C+5% CO_2, cells were stimulated with increasing concentrations of GnRH, buserelin or vehicle control. For antagonistic assays, background signal was obtained with 40 μl culture medium/well and cells were incubated with an excess of the antagonist cetrorelix (160 nM) or vehicle control 30 min prior to stimulation with submaximal (EC_{80}) concentrations of GnRH (31.6 nM) or buserelin (1 nM). For inhibition of the $G\alpha_q$ signaling pathway, αT3-1 cells were pretreated with 1 μM UBO-QIC or vehicle control 30 min prior to stimulation with submaximal (EC_{80}) concentrations of GnRH (31.6 nM) or buserelin (1 nM). Submaximal (EC_{80}) concentrations of GnRH and buserelin were derived from concentration–response curves using Total Area Under the Curve (AUC) analysis (see section 'Section 2.5').

2.4. Inositol phosphate accumulation assay

αT3-1 cells were seeded at a cell density of 100.000 cells/well with [^{3}H]-myoinositol (4 μCi/ml) overnight at 37 °C and 5% CO_2. Subsequently, cells were washed twice with Buffer A containing 127 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, 0.5 mM NaH_2PO_4, 5 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 10 mM HEPES and 0.1% BSA. Thereafter, cells were incubated for 20 minutes at 37 °C with Buffer A supplemented with 50 mM LiCl, followed by stimulation with increasing concentrations of GnRH or buserelin for 60 min at 37 °C. Cells were lysed through 1 h incubation with 10 mM formic acid at 4 °C, after which 20 μL of solution was transferred to an isoplate™ 96 followed by addition of 80 μL YSi Poly-L-Lysine-coated SPA beads at 12 mg/ml. The mixture was shaken at room temperature for 60 min prior to a 5 min centrifuge step at 1500 rpm. Radioactivity of the extract/bead mixture was determined by scintillation spectrometry using the P-E 1450 Micobeta Wallac Trilux scintillation counter according to instruction manual (Perkin Elmer, Groningen, the Netherlands).

2.5. Data analysis

All experimental data were analyzed using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA). Cell index (CI) traces were normalized to time of agonist addition and then exported from RTCA Software 1.2 (Roche, Germany). Total AUC values up to 180 min after agonist addition were used for data analyses. Baseline was removed by subtracting vehicle, antagonist and inhibitor controls from corresponding normalized CI (NCI) traces.

Efficacy (E_{max}) and potency (pEC_{50}) values for GnRH and buserelin were obtained with non-linear regression of Total AUC data fitted by log(agonist) *vs.* response–Variable slope. Results were normalized to Total AUC induced by the maximal concentration of GnRH. Total AUC of agonist-induced cellular responses in presence of antagonist or pathway inhibitor were normalized to Total AUC obtained from αT3-1 cells responses treated with submaximal concentrations (EC_{80}) of corresponding agonist.

Efficacy (E_{max}) and potency (pEC_{50}) values for IP accumulation assay were obtained using non-linear regression of total counts upon GnRH or buserelin-induced $G\alpha_q$-activation fitted by log (agonist) *vs.* response–Variable slope. Results were normalized to total counts induced by the maximal concentration of GnRH.

All values obtained are means ± SEM of at least three independent experiments performed in duplicate.

3. Results

3.1. GnRHR signaling in heterologous and endogenous cells on the xCELLigence

Heterologous GnRH receptor-mediated signaling in CHOhGnRH-NFAT cells was monitored on the xCELLigence system. Overnight proliferation resulted in a cell index of approximately 4.0 (Fig. 1A). Typically, the impedance increased upon agonist addition with a first peak around 10 min of approximately 0.075 NCI, followed by a second peak reaching approximately 0.15 NCI around 80 min. The signal decreased again back to baseline after approximately 180 min (Fig. 1C).

Stimulation of CHOhGnRH-NFAT cells with increasing concentrations of GnRH and its analog buserelin resulted in a concentration-dependent increase in impedance (Fig. 2A and C). From these impedance changes a concentration–response curve could