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Low reversibility of intracellular cAMP accumulation in mouse Leydig tumor cells (MLTC-1) stimulated by human Luteinizing Hormone (hLH) and Chorionic Gonadotropin (hCG)

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ABSTRACT

In order to study the intracellular cAMP response kinetics of Leydig cells to hormones with LH activity, we used MLTC-1 cells transiently expressing a chimeric cAMP-responsive luciferase so that real-time variations of intracellular cAMP concentration could be followed using oxiluciferin luminescence produced from catalyzed luciferin oxidation. The potencies of the different LHs and CGs were evaluated using areas under the curves (AUC) of their kinetics over 60 min stimulation. All mammalian LHs and CGs tested were found to stimulate cAMP accumulation in these cells.

The reversibility of this stimulation was studied by removing the hormone from the culture medium after 10 min of incubation. The ratios of kinetics AUC after removing or not the hormone were used to evaluate the stimulation reversibility of each hormone.

Natural and recombinant hLHs and hCGs were found to exhibit slowly reversible activation compared to pituitary rat, ovine, porcine, camel and equine LHs, serum-derived eCG (PMSG) and recombinant eLH/CGs. Carbohydrate side chains are not involved in this phenomenon since natural and recombinant homologous hormones exhibit the same reversibility rates. It is still unknown whether only one human subunit, α or β, is responsible for this behaviour or whether it is due to a particular feature of the hLH and hCG quaternary structure.

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1. Introduction

Gonadotropins are heterodimeric glycoprotein hormones present only in vertebrates. They are formed by the non-covalent association of one α-subunit common to all glycoprotein hormones in a given species and one hormone-specific β-subunit (Combarnous, 1992; Pierce and Parsons, 1981). Glycoprotein hormones exhibit large structural homologies that can be related to their phylogenies and to the common origin of their α- and β-subunits' ancestors (Alvarez et al., 2009; Dos Santos et al., 2011; Premzl, 2015).

There are three types of glycoprotein hormones based on their specific actions: TSH (Thyroid-Stimulating Hormone) and the gonadotropins LH (Luteinizing Hormone) and FSH (Follicle-Stimulating Hormone) named after their specific functions in the female. The same pituitary gonadotropins are also expressed and active in the male, with LH stimulating Leydig cells and FSH stimulating Sertoli cells.

In two mammalian groups, primates and equidaes, gonadotropins are also synthesized and secreted by the placenta (Chopineau et al., 1997; Martinuk et al., 1991; Ward et al., 1982). The most well known Chorionic Gonadotropins (CG) are the human CG (hCG) (Canfield and Ross, 1976; Cole, 2009) and the equine CG (eCG) (Butnev et al., 1996; Cole, 1936; Stewart and Allen, 1981). These CGs share the same receptors as LHs so that these receptors are named LHCGR in these species. Indeed, hCG and eCG exhibit only LH activity in their species of origin (Combarnous et al., 1978; Stewart and Allen, 1981) although eCG also exhibits FSH activity in many non-equine species but not in the horse (Combarnous, 1992; Combarnous and Henge, 1981; Guillou and Combarnous, 1983).

Abbreviations: CG, Chorionic Gonadotropin; LH, Luteinizing Hormone; MLTC, mouse Tumor Leydig cells; LHR, LH receptor (most species); LHCGR, LH & CG receptor (primates and equidaes).

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Human CG binds to the same receptor as LHs but it has been found several decades ago, to exhibit a slower dissociation rate than ovine LH, from the rat testis LH receptor (Huhtaniemi and Catt, 1981). The two hormones have also been shown to differ in their affinities toward recombinant hLHCG receptors and rat LH receptors (Galet and Ascoli, 2005). In previous reports, we pointed out the hCG "superactivity" in rat Leydig cells compared to other hormones having LH activity (Apparailly and Combarnous, 1994; Combarnous et al., 1986). Superactivity of hCG was defined as its higher ratio of cell-stimulating steroidogenic potency relative to its receptor binding potency compared to several animal LHs.

Although the low reversibility of hCG binding to rat LH receptor was well established, the structural origin of this behaviour was not obvious. Was it due to the carboxy-terminal extension of its β-subunit (generally called carboxy-terminal peptide, CTP) or to other human-specific sequence portions in its α- and β-subunits. To get more insight in this phenomenon we undertook a comparative study of hCG with 1/eLH and eCG that also bear a CTP, 2/hLH that exhibits a higher percentage of identity than non-human LHs with hCG and 3/rat LH that is the closest available substitute for the homologous hormone mouse LH and 4/ovine, porcine, and bovine that are the classical non-human LHs studied so far.

We have used the MLTC-1 cell line transiently transfected with the cAMP Glo-sensor reporter gene in the presence of a phosphodiesterase inhibitor in order to follow LH receptor occupation and binding reversibility through the real-time kinetics of intracellular cAMP accumulation.

2. Experimental procedures

2.1. Hormones

All gonadotropin preparations used were of the highest purity grades available. Highly-purified pituitary hLH-046 (3.8 x NIH LH S1) (Closset et al., 1975) was a kind gift from Dr Jean Closset (University of Liège, Belgium) and highly-purified pituitary hLH-SIAFP1(4500 IU/mg) was provided by Dr AF Parlow (NHPP NIDDK, Torrance, CA, USA). Recombinant hLH-C35 was from Serono (Genève, Switzerland). Natural hCG preparations were obtained from various sources: hCG#8149 (8149 IU/mg) (Istituto Massone, Buenos Aires, Argentina), hCG C120620 (7177 IU/mg) (BBT Biotech GmbH, Baesweiler, Germany), and hCG CY2181 (8390 IU/mg) that was purified in our laboratory from crude material (Diosynth - Akzo Nobel, Oss, The Netherlands). Recombinant hCG C6322 (10,000 IU/mg) was purchased from Sigma (St-Quentin-Fallavier, France). Several other preparations were from NHPP (Torrance, CA, USA): rat LH AFP115368, eCG NIH Standard (PMSG, 2000 IU/vial), bLH USDA-I2 (2.1 x NIH LH S1) and oLH NIH S26 (2.3 x NIH LH S1). Natural eCG (PMSG 7770 IU/mg) was from Syntex (Buenos-Aires, Argentina). The other highly purified natural animal hormones used in the present work were prepared in our laboratory: oLH CY1086 (3.5 x NIH LH S1), pLH CY1235-I (1.2 x NIH LH S1) (Hennen et al., 1971), bLH CY1256 (1.2 x bLH LER1072.2), eLH CY937, natural eCG NZY-01 (Lecompte et al., 1998) and recombinant eLH/CG CC4 produced in our laboratory in baculovirus-Sf9 insect cell system (Legardinier et al., 2008).

Standard PMSGs (natural eCGs) were obtained from NIH (Bethesda, MD, USA) (2000 IU/vial), from ANMAT (Buenos Aires, Argentina) (4600 IU/vial) or from our laboratory (eCG NZY-01 1000 IU/vial) (Lecompte et al., 1998). Commercial eCG preparation was Synchro-Part (6000 IU/vial) from Ceva (Libourne, France).

2.2. MLTC-1 cells

MLTC-1 cells (Rebois, 1982) are murine Leydig cells expressing LH receptors that were obtained from the American Tissue and Cell Collection (ATCC) (LGC Standards, Molsheim, France). Cells were expanded in supplemented RPMI 1640 medium (Gibco, Invitrogen, 10% fetal bovine serum, 50 μg/ml gentamicin, 10 units penicillin/ml and 10 μg/ml streptomycin) and used from passes P6 to P30.

2.3. Culture conditions

One day before each experiment, about 100,000 cells were seeded per well on a 96-well Greiner white/clear bottom plate (Dutscher, Brumath France) and incubated at 37 °C under 5% CO_2 in 200 μl supplemented RPMI growth medium.

Cells were then transfected with pGloSensor™ -22F cAMP plasmid (Promega, France) using XtremeGENE HP DNA transfection reagent (Roche, France). This plasmid consists in firefly luciferase sequence fused to that of the protein kinase A cAMP-binding domain in a way that allows control of its enzymatic activity by cAMP.

Thirty minutes before transfection, DNA (100 ng plasmid per well) and XtremeGENE HP DNA transfection reagent (Roche, France, 0.3 μl per well) were mixed together with serum-free supplemented RPMI medium (10 μl per well). Supernatants were then aspirated from each well and replaced by 100 μl of supplemented RPMI medium plus 10 μl of transfection mix. The plates were then incubated overnight at 37 °C under 5% CO_2 before use of the cells in the assays.

2.4. Assays and measurements

Transfection supernatants in each well were removed and replaced with medium deprived of fetal-calf serum (100 μL) and added with 4μL/well luciferase substrate (GloSensor cAMP Reagent, Promega, France) as well as 1 mM IBMX. Individual stimulating hormones (LH or CG) were added in a 11 μL volume in triplicate wells. When a washing step of the wells was required, recording was suspended, supernatants were eliminated by aspiration, replaced by 110 μl serum-free RPMI medium, then aspirated again and finally replaced by 110 μl equilibrium medium with 1 mM IBMX containing or not the tested molecule. In an alternative method, the medium in control wells are not eliminated.

All the experiments in the present work were carried out using the nucleotide phosphodiesterase (PDE) inhibitor IBMX to suppress any effect on this enzyme and to concentrate our study on LH- and CG-stimulated cAMP synthesis only, so the recorded response is more directly related to LH receptor occupation.

In all experiments, time courses of luminescence were recorded over 60 min at 28 °C in triplicate wells for each condition using a POLARstar Omega automated microplate reader (BMG Labtech, Offenburg, Germany). Means and standard deviation (SD) values were calculated from each triplicate luminescence recordings at each time point of the kinetics. Dose-dependent curves were derived from these luminescence kinetics by plotting Areas Under Curve (AUC) as a function of hormone concentration.

2.5. Area Under Curve (AUC) calculations and statistical analyses

The GraphPad 5.00 package (GraphPad Software, San Diego CA, USA) was used for Area Under Curve (AUC) determinations of individual kinetics as well as for slope calculation by linear fitting of initial accumulation rate. Mean and SD values for each triplicate AUCs were determined. The AUC ratios were used to compare cAMP response kinetics after hormone removal at 10 min vs control kinetics where hormone concentrations were kept constant over the whole kinetics.

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