Steroids 100 (2015) 21–26

Contents lists available at ScienceDirect

Steroids

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

Cell-based assay of nongenomic actions of progestins revealed inhibitory G protein coupling to membrane progestin receptor α (mPRα)

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ARTICLE INFO

Article history:
Received 23 November 2014
Received in revised form 13 March 2015
Accepted 14 April 2015
Available online 21 April 2015

Keywords:
Membrane progestin receptor
Luciferin
Diethylstilbestrol (DES)
Endocrine disruptor
Nongenomic steroid action
Goldfish

ABSTRACT

Previously, we established cell lines stably producing goldfish membrane progestin receptor α (goldfish mPRα) proteins, which mediate steroidal nongenomic actions. In this study, we transfected these cell lines (MDA-MD-231) with cDNAs encoding a recombinant luciferase gene (GloSensor). These cells can be used for monitoring the effects of ligands that bind to mPR by means of luminescence, the intensity of which reflects intracellular cyclic adenosine monophosphate (cAMP) levels.

Luminescence intensity of the cells increased significantly when cells were treated with forskolin, strong activator of adenylyl cyclase. Then, we established a strategy to measure changes in luminescence that correlated with the actions of the ligands. The actions of ligands were measurable by the prevention of stimulation caused by forskolin after ligand stimulation.

The studies using these cell lines indicated that cAMP concentrations were decreased specifically by the mPR ligands 17α,20β-dihydroxy-4-pregnen-3-one, diethylstilbestrol and progesterone. Furthermore, pertussis toxin inhibited the decrease in cAMP levels caused by mPR ligands. These results support evidence from previous results that mPRα is coupled to an inhibitory G protein.

1. Introduction

Rapid nongenomic effects of progestin and membrane progestin receptor (mPR) expression have been investigated in a wide variety of tissues of vertebrates. mPR, which mediates the nongenomic actions of progestin on the plasma membrane, was first identified in teleosts and subsequently in other vertebrates, including humans [1,2]. Research on the functions of mPR have identified it as a mediator of maturation-inducing hormones (MIHs), which induce oocyte maturation (OM) in fish [3–6]. Although mPRs have been shown to mediate nongenomic steroid actions through an inhibitory G protein (Gi) and by inducing a decrease in intracellular cyclic adenosine monophosphate (cAMP) levels, this evidence is currently restricted to biochemical studies [7]. Thus, evidences from different angles are necessary for the molecular mechanisms associated with mPRs. In this study, the ability of ligands binding to mPR to cause a decrease in cAMP levels was investigated in cell-based assays using cancer cells transfected with mPRα and recombinant luciferase (GloSensor), which could be activated by cAMP. GloSensor-transfected cells showed high luminescence after stimulation with forskolin, a strong activator of adenylate cyclase. Agonists for mPR, 17α,20β-dihydroxy-4-pregnen-3-one (17,20β-DHP), progesterone (P4) and diethylstilbestrol (DES), which bind to mPR, induced decreases in intracellular cAMP levels, whereas 17β-estradiol (E2), testosterone and androstenedione showed almost no activity in this GloSensor cell line. The close correspondence between the binding of a ligand to mPRα and their cAMP-decreasing activities suggested a mechanism of signal

Abbreviations: 17,20β-DHP, 17α,20β-dihydroxy-4-pregnen-3-one; ANOVA, analysis of variance; cAMP, cyclic adenosine monophosphate; DES, diethylstilbestrol; E2, 17β-estradiol; FBS, fetal bovine serum; Gi, inhibitory G protein; MIH, maturation-inducing hormone; mPR, membrane progestin receptor; OM, oocyte maturation; P4, progesterone; PTX, pertussis toxin.

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http://dx.doi.org/10.1016/j.steroids.2015.04.002
0039-128X/

transduction by binding to mPRα resulting in the inhibition of adenylate cyclase through a coupled Gi.

2. Experimental

2.1. Materials

Androstenedione, 17,20β-DHP, DES, P4, testosterone and 17β-estradiol were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Cell culture and transfection

Human MDA-MB-231 breast carcinoma cells (American Type Culture Collection) stably transfected with goldfish mPRα were transfected again with recombinant luciferase that could be activated by cAMP (GloSensor; Promega, Madison, WI, USA), as described previously [8]. The cells were cultured and maintained at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (Sigma) containing 5% fetal bovine serum (FBS; Gibco, Carlsbad, CA, USA) and 100 μg/mL gentamicin (Invitrogen, Carlsbad, CA, USA). The medium was changed every 2 days, and cells were split among three plates when they became 90% confluent. Purified vector constructs of GloSensor were then transfected into the cells using Lipofectamine 2000 reagent (Invitrogen) in accordance with the manufacturer's instructions. Two days after transfection, plasmid-expressing cells were selected using 200 μg/mL hygromycin B (Invitrogen). Resistant colonies were propagated with 200 μg/mL hygromycin B in order to produce stably transfected cell lines. Finally, transfected cells were cloned by the limiting dilution method.

2.3. Luminescence assays

For culture the cells out of CO_2 incubator during the assay, the medium contains a unique buffering system composed of mono and dibasic sodium phosphate and β-glycerophosphate that can support the cells without an exogenous source of CO_2 (CO_2-independent medium) was used. The day before the assay, 8×10^4-cells/well were split into 96 well plates. Next day, the culture medium was carefully removed and 35 μL of CO_2-independent medium added as equilibration medium containing 2% v/v dilution of GloSensor™ cAMP Reagent stock solution and 0.001% Tween 80. Then, the plate was set in a luminescence detector (Luminescensor JNRII, ATTO, Tokyo, Japan). Detection was started immediately and continued for 30 min to 2 h or until a steady-state basal signal was obtained at 25 °C. The luminescence intensity of each well was measured every 5 min. Then, the plate was removed from the detector and the medium was exchanged to CO_2-independent medium without luciferin and Tween 80 (125 μL per well) containing ligands dissolved in ethanol. The plate was set again in a luminescence detector and detection was started. The luminescence intensity of each well was measured every 5 min. After 5–15 min, the plate was removed from the detector and 25 μL of forskolin stock solution (60 μM in CO_2-independent medium) was added. The plate was returned to the detector. The luminescence intensity of each well was measured every 5 min. We found no deleterious effects associated with running the assays using a final concentration of 1% ethanol.

2.4. Statistical analysis

All experiments were repeated at least three times. One-way analysis of variance (ANOVA) of the data was analyzed using the GraphPad Prism Program (San Diego, CA, USA). A P value <0.05 was considered statistically significant.

3. Results

3.1. Establishment of mPRα- and GloSensor-transfected cells

We had previously established cultured cells expressing the recombinant goldfish mPRα protein [8]. The protein showed specific binding activity with mPR ligands. To establish cells to enable the measurement of intracellular cAMP levels after stimulation by mPR ligands, the cells were transfected with genes encoding recombinant luciferase proteins (GloSensor). Recombinant luciferase, including the cAMP binding domain from the regulatory subunit of human protein kinase A, degrade luciferin only in the presence of cAMP. The features of the protein make it possible to sense intracellular cAMP by means of luminescence. Selection of transfectants used hygromycin B for 2 months after transfection, and expression of mRNA for GloSensor protein was confirmed by RT-PCR (Fig. 1A). In order to examine the activity of GloSensor-transfected cells, changes in luminescence intensity after stimulation with forskolin were measured using a luminescence detector. Although luminescence could not be measured in accordance with the manufacturer's instructions established for a HEK 293 cell line, we found that detergents could support the penetration of luciferin into the cells (Fig. 1B). Among the detergents tested, Tween 80 was the most effective to achieve higher luminescence intensity. Thus, we selected Tween 80 as the detergent in our method. Then, we observed the luminescent cells. As expected, almost all the cells showed luminescence after stimulation by forskolin (Fig. 1C). Expressions of recombinant proteins in the cells were confirmed by Western blotting (Fig. 1D). A distinct band at approximately 76 kDa was present in the soluble proteins extracted from the GloSensor-transfected cells. 47 kDa band of mPRα was also detected in the membrane proteins as previously described [8]. From these results, we concluded that the transfection was successful, and functional recombinant luciferases were produced in the cells.

3.2. Establishment of assay protocol by using transfected cells

We attempted to detect the actions of ligands by adding only the ligands directly into medium. We selected DES as the standard agonist for goldfish mPRα (Fig. 2A). Although a slight decrease in luminescence was detectable in the highest concentration of DES, it was hard to measure the difference exactly. Then, we tried to measure the inhibitory effect of the G protein, which was stimulated by ligands binding to mPRα by using forskolin, as an activator of adenylate cyclase. Forskolin was added into the medium after the addition of ligands (Fig. 2B). When luminescence was stimulated by forskolin in the ethanol-treated cells, the magnitude of stimulation was decreased in DES-treated cells. At the highest concentration of DES, the increase in luminescence almost fully inhibited. We set the margin between the luminescence from the ethanol-treated cells and that from ligand-treated cells as the activity of the ligand (Fig. 2B). We concluded that we could establish a cell-based assay for the nongenomic actions involving mPR protein by measuring changes in intracellular cAMP levels.

3.3. Specific actions of mPR ligands on decreases in cAMP levels

We evaluated the specificity of the assay system by examining ligand specificity. We analyzed responses to steroids and DES (Fig. 3). Among the compounds tested, only 17,20β-DHP, P4 and DES as mPR ligands were effective for inhibiting forskolin-induced