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The $G\alpha_{q/11}$-provoked induction of *Akr1c18* in murine luteal cells is mediated by phospholipase C

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ABSTRACT

Towards the end of gestation prostaglandin F2α (PGF2α) stimulates the expression of *Akr1c18* in the murine corpus luteum. *Akr1c18* codes for 20α-hydroxysteroid dehydrogenase, an enzyme that precipitates parturition by catabolizing progesterone.

Previous results from our laboratory have shown that this effect of PGF2α is mediated by the activation of $G\alpha_{q/11}$, but the downstream effector(s) of $G\alpha_{q/11}$ that elicit the increase in *Akr1c18* expression have not been identified. The physiological effects of $G\alpha_{q/11}$ are mediated by its ability to interact with phospholipase Cβ, p63RhoGEF, and PKCζ. In the experiments described herein we used biochemical and pharmacological approaches, as well as adenoviral-mediated expression of a constitutively active form of $G\alpha_q$ and mutants thereof, to examine the role of each of these effectors as potential mediators of the increased expression of luteal *Akr1c18*.

By measuring the effects of PGF2α on the activation of RhoA (activated by p63RhoGEF) and the effects of activators and inhibitors of RhoA on the PGF2α-induced expression of luteal *Akr1c18*, we determined that RhoA is neither activated by PGF2α or involved in the PGF2α-induced expression of luteal *Akr1c18*. The potential involvement of PKCζ was ruled out by the inability of a mutant of a constitutively active $G\alpha_q$ that prevents PKCζ binding to block the increased expression of *Akr1c18*. Furthermore, PGF2α does not increase the phosphorylation of ERK-5, the only known downstream target of PKCζ. On the other hand, three different mutants of a constitutively active $G\alpha_q$ that prevent phospholipase C activation blocked the induction of luteal *Akr1c18*. We conclude that the induction of luteal *Akr1c18* by $G\alpha_{q/11}$ is mediated by the activation of phospholipase C.

1. Introduction

The $G_{q/11}$ family of G proteins expressed in murine granulosa/luteal cells plays two important roles in female fertility. First, upon activation by the lutropin/choriogonadotropin receptor (LHR), the granulosa cell $G\alpha_{q/11}$ as well as the granulosa cell $G\alpha_s$ are both required for full induction of the granulosa cell progesterone receptor, an event required for the rupture of ovulating follicles (Breen et al., 2013). Second, upon activation by the agonist-engaged prostaglandin F2α receptor at the end of gestation, the luteal cell $G\alpha_{q/11}$ are essential for the induction of *Akr1c18*, the gene coding for 20α-hydroxysteroid dehydrogenase (Mejia et al., 2015), an enzyme that precipitates parturition by catalyzing a reduction in progesterone levels (Stocco et al., 2007). In addition to *Akr1c18*, the prostaglandin F2α receptor-induced activation of luteal $G_{q/11}$ at the end of gestation also impacts the expression of many other luteal genes (Waite et al., 2016).

The classical target for activated $G\alpha_{q/11}$ is phospholipase Cβ. The activation of this enzyme leads to an increase in the conversion of phosphatidylinositol 4,5-bisphosphate to 2 s messengers, inositol 1,4,5 trisphosphate and diacylglycerol, each of which impact a variety of cellular functions (Berridge, 1987; Exton, 1996; Venkatakrishnan and Exton, 1996; Waldo et al., 2010). More recent studies, however, have identified three other partners for $G\alpha_{q/11}$ with unique contributions to its signaling properties (reviewed in Sanchez-Fernandez et al., 2014). The guanine nucleotide exchange factor p63RhoGEF is a $G\alpha_{q/11}$ effector that activates RhoA, a small G protein (Lutz et al., 2005, 2007; Rojas et al., 2007). Protein kinase Cζ (PKCζ) binds to $G\alpha_{q/11}$ and participates in the phosphorylation/activation of

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ERK-5 through the formation of a ternary complex containing $G\alpha_{q/11}$ and ERK-5 bound to PKCζ (Garcia-Hoz et al., 2010; Sanchez-Fernandez et al., 2016). Finally, G protein-coupled receptor kinase 2 (GRK2) is a $G\alpha_{q/11}$ partner that attenuates signaling by inhibiting the ability of $G\alpha_{q/11}$ to bind phospholipase Cβ (Day et al., 2003; Sterne-Marr et al., 2003; Tesmer et al., 2005) and PKCζ (Sanchez-Fernandez et al., 2016). In view of the multitude of effectors that mediate the actions of $G\alpha_{q/11}$ it is important to identify the effectors that mediated the PGF2α-induced increase in *Akr1c18* to fully understand the molecular basis of the induction of this important ovarian gene but also to understand the actions of PGF2α and $G\alpha_{q/11}$ in general. A previous study suggested the involvement of phospholipase C because the PGF2α-induced expression of *Akr1c18* in luteal cells was shown to be inhibited by calcium/calmodulin inhibitors (Stocco et al., 2002). On the other hand, not all actions of $G\alpha_{q/11}$ are dependent on phospholipase C as documented by the involvement of Rho activation on the induction of uterine contractions by PGF2α/$G\alpha_{q/11}$ (Goupil et al., 2010) and the involvement of PKCζ on the induction of apoptosis by carbachol/$G\alpha_{q/11}$ (Sanchez-Fernandez et al., 2016).

The experiments described herein were designed to identify which of the different $G\alpha_{q/11}$ binding partners discussed above mediate the PGF2α/$G\alpha_{q/11}$-mediated induction of the luteal *Akr1c18* (Mejia et al., 2015). To address this question, we measured the effects of PGF2α on two downstream effectors of $G\alpha_{q/11}$ (RhoA and ERK-5) and the effects of activators and inhibitors of RhoA on the PGF2α-induced expression of luteal *Akr1c18*. We also determined the effects of adenoviral-mediated expression of several mutants of activated $G\alpha_q$ that prevent the interaction of $G\alpha_q$ with each of its effectors on the induction of luteal *Akr1c18*.

2. Materials and methods

2.1. Adenoviral constructs

The preparation of the recombinant adenoviral particles coding for β-galactosidase (Ad-βgal, used as control) and a constitutively active mutant (Q209L) of the human $G\alpha_q$ that cannot hydrolyze GTP have been described (Donadeu and Ascoli, 2005). The original human $G\alpha_q$Q209L construct subcloned in pcDNA3.1 was purchased from the cDNA resource center (http://www.cdna.org), and it was also used to construct 3 mutants (designated mt1, mt2 and mt3) using the Quick Change Site Directed Mutagenesis kit from Agilent technologies (http://www.agilent.com). Mt1 refers to $G\alpha_q$(Q209L,-R256A,T257A), a constitutively active form of $G\alpha_q$ that is unable to activate phospholipase Cβ (Fan et al., 2003; Venkatakrishnan and Exton, 1996). Mt2 refers to $G\alpha_q$(Q209L,Y261F,W263D), a constitutively active mutant of $G\alpha_q$ that is unable to bind GRK2 (Tesmer et al., 2005), and mt3 refers to $G\alpha_q$(Q209L,E234A,E245), a constitutively active mutant of $G\alpha_q$ that is unable to bind PKCζ (Sanchez-Fernandez et al., 2016). Recombinant adenoviral particles coding for each of these three mutants were prepared by Applied Biological Materials (https://www.abmgood.com).

2.2. Mice

A colony of mice harboring a granulosa/luteal cell specific deletion of *Gnaq* in the context of a global deletion of *Gna11* (*Gnaq*$^{f/f}$;*Gna11*$^{-/-}$;*Cre*$^{+}$) was generated by crossing *Gnaq*$^{f/f}$;*Gna11*$^{-/-}$ mice and *Cyp19Cre* transgenic mice as previously described (Breen et al., 2013). This colony was then maintained and genotyped by crossing *Gnaq*$^{f/f}$;*Gna11*$^{-/-}$;*Cre*$^{+}$ males with *Gnaq*$^{f/f}$;*Gna11*$^{-/-}$;*Cre*$^{-}$ females as described (Mejia et al., 2015). The resulting *Gnaq*$^{f/f}$;*Gna11*$^{-/-}$;*Cre*$^{+}$ and *Gnaq*$^{f/f}$;*Gna11*$^{-/-}$;*Cre*$^{-}$ females were used as experimental and control animals, respectively (Mejia et al., 2015).

Immature female mice (21–24 days old) were injected intraperitoneally with 5 IU pregnant mare serum gonadotropin (PMSG; National Hormone and Peptide Program) to induce follicular growth. Forty-four to 48 h later they received an intraperitoneal injection of 5 IU human chorionic gonadotropin (hCG, Sigma Chemicals) to induce ovulation and luteinization. To maintain the corpora lutea, mice received two daily intraperitoneal injections of 2.5 IU ovine prolactin (Sigma Chemicals) at 0900–1000 and at 1700–1800 h for two consecutive days starting 24 h following the injection of hCG. Ovaries were then obtained from animals that were sacrificed 18 h after the last prolactin injection. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Iowa.

2.3. Luteal cell cultures, transductions and assays

The corpora lutea were teased apart from the ovaries with 21 gauge needles and luteal cells were isolated by digestion with collagenase and DNAse using slight modifications (Mejia et al., 2015) of a previously published method (Thordarson et al., 1997). Luteal cells were counted and plated (day 1) at a ratio of 1 mouse/well (5–7 x 10^5 cells) into each well of a 24-well plate using a total volume of 500 μl of DMEM/F12 supplemented with 10 mM Hepes, bovine serum albumin (1 mg/ml), insulin (1 μg/ml), transferrin (1 μg/ml), selenium (1 ng/ml), gentamicin (50 μg/ml), fungizone (2.5 μg/ml) and ovine prolactin (2.5 mIU/ml) (henceforth referred to as complete medium).

The cells were allowed to attach for 18–24 h at 37C and the wells were washed twice on day 2 with medium containing 1 mg/ml bovine serum albumin and incubated again for another 24 h in 500 μl of complete medium prior to any additional manipulations. Alternatively, on day 2 the cells were washed twice with medium without bovine serum albumin and then transduced with Ad-βgal (control), Ad-GαqQ209L (henceforth referred to as Q209L) or one of the three different mutants described above (mt1, mt2 or mt3) in complete medium, but devoid of bovine serum albumin. The amount of each recombinant adenovirus used for transduction varied and was chosen empirically to equalize their expression as documented in Fig. 5. The total viral load, however, was maintained at a constant MOI of 200 by adding varying amounts of Ad-βgal. After a 2 h incubation the virus-containing medium was removed and replaced with complete medium and the cells were incubated for an additional 18–24 h prior to any further manipulations.

To measure the phosphorylation of ERK-5 and ERK-1/2 or the activation of RhoA, the cells were washed twice on day 3 with medium with 1 mg/ml bovine serum albumin and incubated in 500 μl of medium with 1 mg/ml bovine serum albumin for 18–24 h. At this point (day 4) the medium was replaced with 500 μl of medium with 1 mg/ml bovine serum albumin and the cells were stimulated as indicated in the figure legends prior to the preparation of lysates. Lysates for Western blots were prepared as described elsewhere (Breen et al., 2013), and lysates for the RhoA assays were prepared exactly as described by the manufacturer of a kit designed to measure RhoA activation (catalog # BK124 from cytoskeleleton.com). Assays for activated RhoA were done using 50–70 μg of protein exactly as described by the manufacturer (catalog # BK124 from cytoskeleleton.com). The phosphorylation of ERK-5 or ERK-1/2 were ascertained using Western blots loaded with 2–10 μg of protein per lane. The gels used to prepare the blots developed with PERK-5, PERK-1/2 or ERK-1/2 antibodies were the standard gels. The gels used to prepare the blots developed with ERK-5 antibodies were run using Phos-tag™(Wako Chemicals) to allow for a better separation between phosphorylated and unphosphorylated proteins by mobility shifts (Kinoshita et al., 2006). P-ERK-5 and ERK-5 antibodies were from Millipore (cat #07–507) and Sigma (cat

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