Endocrine, *vol. 8, no. 3, 301–307, June 1998* 0969–711X/98/8:301–307/$9.75

Stepwise Activation of the Gonadotropic Signal Transduction Pathway, and the Ability of Prostaglandin $F_{2\alpha}$ to Inhibit This Activated Pathway

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Through selective activation of the gonadotropic signal transduction pathway, we have determined the probable site of the antigonadotropic effects of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) in the human granulosa-luteal cell (hGLC). The gonadotropic signal transduction pathway was activated at the level of the receptor (luteinizing hormone and β-adrenergic), stimulatory G protein (G_s), adenylate cyclase (AC), and protein kinase A (PKA) by human chorionic gonadotropin (hCG) and isoproterenol (Iso), cholera toxin (CTX), forskolin, and dibutryl cAMP (Db cAMP), respectively. Concomitantly, the ability of $PGF_{2\alpha}$ to inhibit progesterone production in response to the activation of this cascade at these different levels was examined. hGLCs were obtained from in vitro fertilization patients and were precultured for 8 d in Medium 199 supplemented with fetal bovine serum (M199; 10% FBS). Following the preculture period, cells were treated with either vehicle or one of the above activators of the gonadotropic pathway, either in the absence or presence of $PGF_{2\alpha}$ (in M199; No FBS). Following the treatment period, media were collected and assayed for progesterone by RIA. Prostaglandin $F_{2\alpha}$ (10^{-6} *M*) significantly inhibited hCG (1 IU/mL), Iso (10^{-5} *M*), CTX (1 μg/mL), and forskolin- (10^{-5} *M*) stimulated progesterone production. Conversely, $PGF_{2\alpha}$ did not inhibit progesterone production stimulated by a saturating concentration of Db cAMP (10^{-6} *M*). The ability of $PGF_{2\alpha}$ to inhibit hCG- or CTX-stimulated progesterone production was attenuated by pertussis toxin (PTX; 50 ng/mL). In conclusion, through a pertussis toxin-sensitive G protein, $PGF_{2\alpha}$ inhibits progesterone production at a level below AC, and above the activation of PKA by cAMP.

Received December 3, 1997; Revised March 23, 1998; Accepted March 23, 1998.

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Key Words: Prostaglandin $F_{2\alpha}$; human; granulosa; luteal; G protein.

Introduction

In humans and other mammals, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) has been demonstrated to be antigonadotropic (luteolytic), or at times, luteotropic *(1–8)*. The antigonadotropic actions of $PGF_{2\alpha}$ are through the inhibition of luteinizing hormone (LH)-, human chorionic gonadotropin- (hCG), or prostaglandin E_2- (PGE_2) stimulated cAMP and progesterone production, and/or an increase in progesterone catabolism through 20-α-hydroxysteroid dehydrogenase *(5)*. Additionally, $PGF_{2\alpha}$ has been shown to increase cAMP-phosphodiesterase (PDE) activity, via activation of protein kinase C (PKC), in the human granulosa cell *(1)*.

Exposure of human, primate and rodent granulosa/luteal cells to $PGF_{2\alpha}$ is known to activate phospholipase C and its downstream pathways *(9–13)*, as well as inhibit hCG- and gonadotropin-stimulated cAMP production *(1,9,14,15)*. However, several reports have demonstrated the antigonadotropic effects of $PGF_{2\alpha}$ in the presence of inositol phosphate, calcium, and calmodulin inhibitors *(14–17)*, suggesting that activation of the PLC pathway may not be necessary for this effect. At present, this enigma remains unsolved.

Prostaglandin $F_{2\alpha}$-receptor cDNA sequence analysis has suggested a G-protein-coupled receptor *(18,19)*, as has been found with other cloned prostanoid receptors *(20–23)*. However, until now, pharmacological studies confirming G-protein involvement in the actions of $PGF_{2\alpha}$ had not been done in the human ovarian cell. Immunocytochemical studies have localized four different G-protein α-subunits to the hGLC, including: $G_{\alpha s}$, $G_{\alpha i3}$, $G_{\alpha i1,2}$, and $G_{\alpha p}$ (namely $G_{\alpha q}$ and $G_{\alpha 11}$) *(24)*. Furthermore, it has been demonstrated in these cells that cAMP production is regulated by the ratio of $G_{\alpha s}$ and $G_{\alpha i}$, whereas the production of inositol phosphates and rises in intracellular calcium concentration appear to be regulated by $G_{\alpha p}$ ($G_{\alpha q}$ and $G_{\alpha 11}$) and $G_{\alpha i}$ *(24)*.

$PGF_{2\alpha}$ has been shown to lower gonadotropin- and prostaglandin E_2 (PGE_2)-stimulated progesterone production

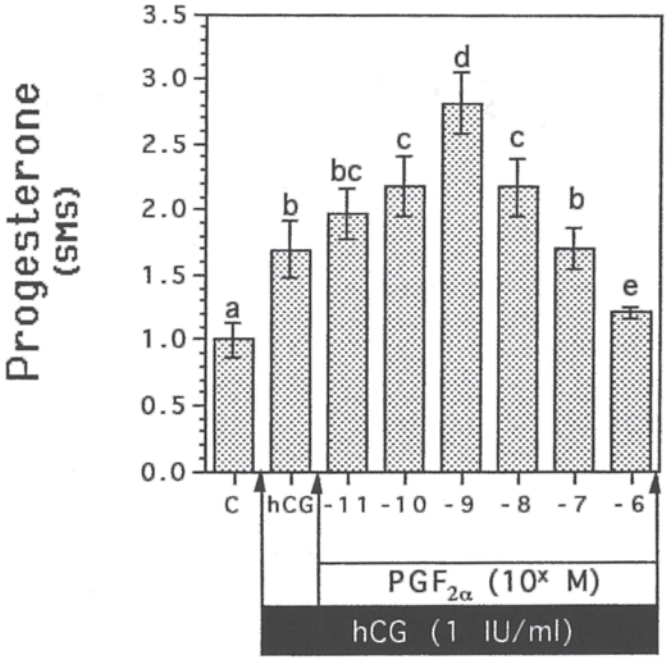

Fig. 1. Progesterone response (over 24 h) to $PF_{2\alpha}$ (10^{-11}–10^{-6} *M*) in the presence of hCG (1 IU/mL), in 8-d cultured hGLCs. Different characters above graph bars signify significant difference ($p < 0.05$ by ANOVA). Data represent the mean ± SEM of standard mean scores (SMS) from six separate experiments performed on cells from separate patients.

through a lowering of cAMP levels. Thus, in order to determine the site of action of $PGF_{2\alpha}$ (with respect to its functionally luteolytic effects), we activated the gonadotropic signal transduction pathway in a stepwise fashion at the level of the receptor (hCG, isoproterenol [Iso]), G protein (cholera toxix [CTX]), adenylate cyclase (AC) (forskolin), and protein kinase A (PKA) (dibutyryl cAMP [Db cAMP]), in the absence or presence of $PGF_{2\alpha}$. Additionally, since G proteins are known to regulate cAMP levels within these cells, PTX and CTX were utilized to elucidate the potential role of G proteins in the antigonadotropic actions of $PGF_{2\alpha}$.

Results

Selection of an Antigonadotropic Concentration of $PGF_{2\alpha}$

In the presence of hCG, concentration-dependent changes in the progesterone response to $PGF_{2\alpha}$ were observed (Fig. 1; $n = 6$). $PF_{2\alpha}$ (10^{-6} *M*) inhibited hCG-stimulated progesterone production in 8-d- (d 8) cultured granulosa-luteal cells (GLCs). In contrast, $PGF_{2\alpha}$ (10^{-10}–10^{-8} *M*) potentiated hCG-stimulated progesterone production in d 8-cultured GLCs. A similar trend was seen for estradiol production (not shown). For this reason, 10^{-6} *M* was selected as a suitable concentration for examining the functional luteolytic effects $PGF_{2\alpha}$ for the remaining experiments.

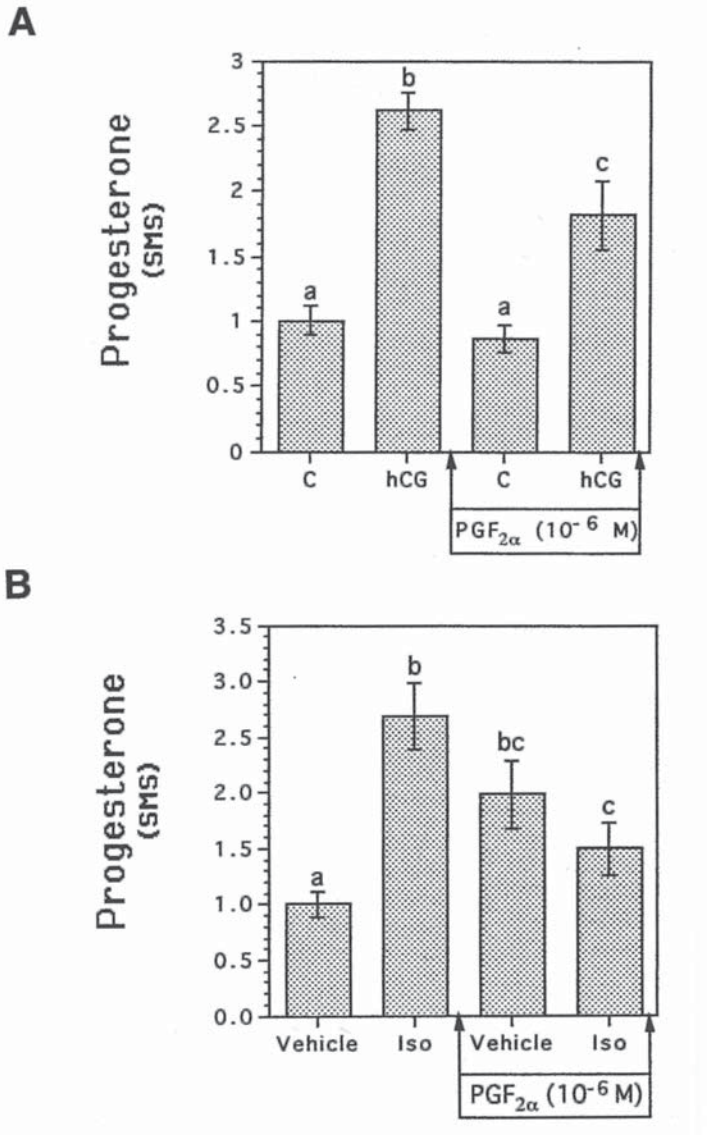

Fig. 2. $PF_{2\alpha}$- (10^{-6} *M*) mediated inhibition of **(A)** (hCG; 1 IU/mL) and Iso- **(B)** (10^{-5} *M*) stimulated progesterone production (over 24 h), in 8-d cultured hGLCs. Data represent the mean ± SEM of SMS from four separate experiments performed on cells from separate patients. Different characters above graph bars signify significant difference ($p < 0.05$ by ANOVA).

Stepwise Activation of the Gonadotropic (Receptor-G_s-AC-cAMP-PKA) Signal Transduction Pathway, and the Ability of $PGF_{2\alpha}$ to Inhibit This Activated Pathway

Stimulation of progesterone production at the receptor level: Progesterone production was significantly stimulated by hCG (Fig. 2A) and Iso (Fig. 2B), in 8-d cultured hGLCs. In cells that were exposed to hCG and $PGF_{2\alpha}$ (Fig. 2A) or Iso and $PGF_{2\alpha}$ concomitantly (Fig. 2B), progesterone production was attenuated. Similar responses were seen with estradiol production (not shown). Thus, $PGF_{2\alpha}$ can inhibit receptor-mediated progesterone production.

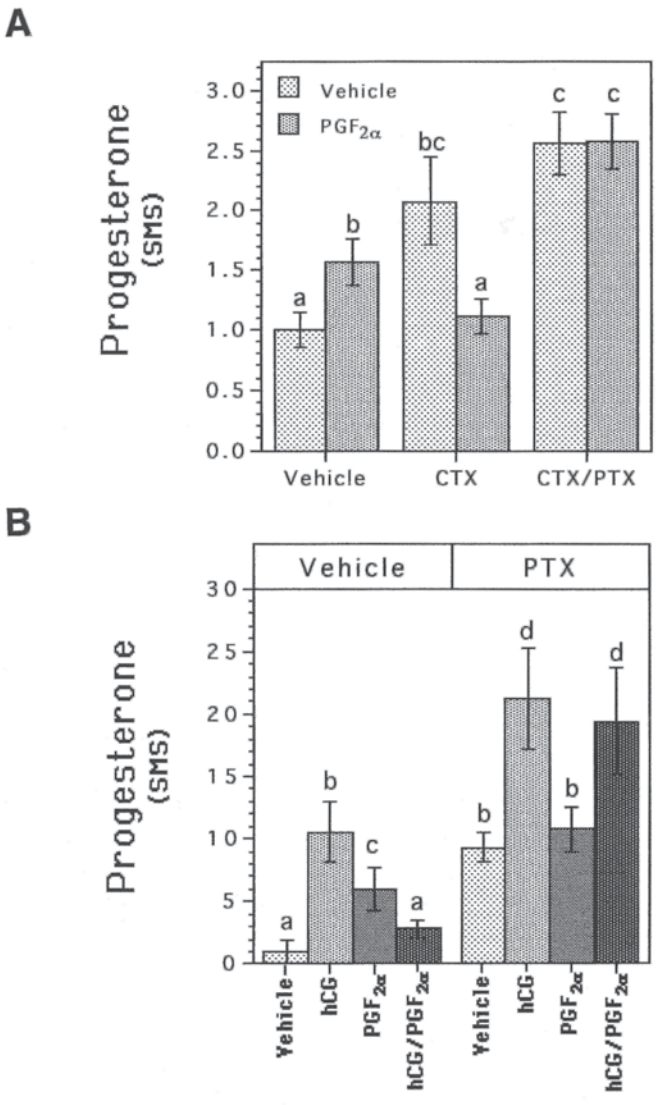

Fig. 3. (A) The effects of $PF_{2\alpha}$ (10^{-6} *M*) on CTX (1 µg/mL) and CTX plus PTX (50 ng/mL) stimulated progesterone production (over 24 h). **(B)** The effects of PTX (50 ng/mL) on $PF_{2\alpha}$- (10^{-6} *M*) mediated inhibition of hCG (1 IU/mL) stimulated progesterone production (over 24 h). Data represent the mean ± SEM of SMS from five separate experiments, performed on 8-d cultured hGLCs, from five different patients. Different characters above graph bars signify significant difference ($p < 0.05$ by ANOVA).

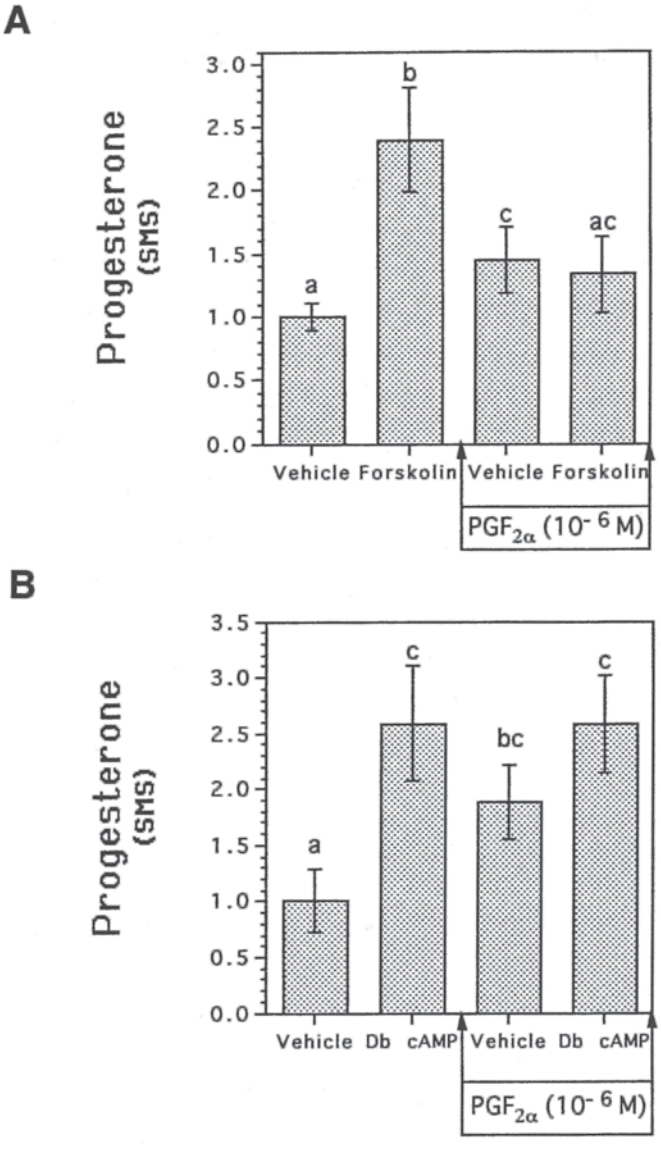

Fig. 4. $PF_{2\alpha}$- (10^{-6} *M*) mediated inhibition of forskolin **(A)** (10^{-6} *M*) and DbcAMP- **(B)** (10^{-5} *M*) stimulated progesterone production, in 8-d cultured hGLCs. Data represent the mean ± SEM of SMS from three (A) or four (B) separate experiments performed on cells from separate patients. Different characters above graph bars signify significant difference ($p < 0.05$ by ANOVA).

Stimulation of progesterone production at the G-protein level: Cholera toxin also significantly stimulated progesterone production from hGLCs, whereas the coapplication of $PGF_{2\alpha}$ significantly blocked this response (Fig. 3A). Interestingly, pertussis toxin (PTX) reversed the ability of $PGF_{2\alpha}$ to inhibit cholera toxin- (CTX)-stimulated progesterone production. Similar responses were seen with estradiol production (not shown). Thus, $PGF_{2\alpha}$ can inhibit G_s-mediated progesterone production.

Stimulation of progesterone production at the level of adenylate cyclase (AC): Progesterone production was significantly stimulated by forskolin (Fig. 4A). Forskolin-stimulated progesterone production was inhibited by coincubation of cells with $PGF_{2\alpha}$ (Fig. 4A). Thus, irreversibly activated AC-mediated progesterone production can be inhibited by $PGF_{2\alpha}$.

Stimulation of progesterone production by the activation of protein kinase A (PKA): On the contrary, $PGF_{2\alpha}$ had no effect on progesterone production, which was significantly stimulated by an excess of Db cAMP (Fig. 4B). Similar results were seen when Db cAMP was replaced with either 8-bromo-cAMP or Sp-cAMPs (not shown). Thus,

Table 1
Stepwise Activation of the Gonadotropic Signal Transduction Pathway:
A Method for the Identification of the Site of $PGF_{2\alpha}$-Mediated Functional Luteolysis[a]

Steroidogenic agent, SA	Target	Second drug	Second target	Progesterone production, PP owing to SA	$PGF_{2\alpha}$ inhibits stimulated PP
hCG	LH/hCG-R	—	—	Present	Yes
Isoproterenol	βReceptor	—	—	Present	Yes
CTX	G_s	—	—	Present	Yes
Forskolin	AC	—	—	Present	Yes
Db cAMP (excess)	PKA	—	—	Present	No
hCG	LH/hCG-R	—	—	Present	Yes
hCG	LH/hCG-R	PTX	G_i, G_p[b]	Present	No
CTX	G_s	PTX	G_i, G_p[b]	Present	No

[a]LH/hCG-R, luteinizing hormone/human chorionic gonadotropin receptor; βReceptor, β-adrenergic receptor; hCG, human chorionic gonadotropin; AC, adenylate cyclase; PKA, protein kinase A; PTX, pertussis toxin; CTX, cholera toxin; G_s, stimulatory G proteins; G_i, inhibitory G-proteins; G_p, phosphoinositide phosphodiesterase activating G proteins.
[b]NB: Some isoforms of Gp are PTX-sensitive, but others are PTX-insensitive.

$PGF_{2\alpha}$ was unable to inhibit progesterone production stimulated by activated PKA. For a summary of these results, please *see* Table 1.

The Role of G Proteins in $PGF_{2\alpha}$-Mediated Functional Luteolysis

The treatment of 8-d cultured hGLCs with hCG (1 IU/mL) significantly stimulated progesterone production (Fig. 3B). As before, this stimulation was inhibited by cotreatment with $PGF_{2\alpha}$. However, in the presence of PTX, this $PGF_{2\alpha}$-mediated inhibition of hCG-stimulated progesterone production was significantly blocked. Similar responses were seen from 1-d cultured cells. Moreover, similar responses were seen for estradiol production (not shown). Thus, $PGF_{2\alpha}$-mediated functional luteolysis appears to be exerted through a PTX-sensitive G-protein. For a summary of these results, please *see* Table 1.

DNA Levels in Response to Treatments

When compared with a vehicle control, the DNA levels of d 8 GLCs remained unchanged by an incubation (24 h) with $PGF_{2\alpha}$, CTX, PTX, or CTX plus PTX, either in the absence or presence of hCG (not shown; $n = 3$). These results suggest that the responses observed were owing to alterations in steroid production rather than changes in the number of cells per well.

Discussion

In the present study, we sought to elucidate the site of action of $PGF_{2\alpha}$ with respect to its functionally luteolytic effects. This was achieved by selectively activating the gonadotropic signal transduction pathway at progressively lower and lower levels, and observing the ability of $PGF_{2\alpha}$ to block progesterone production. First, a functionally luteolytic concentration of $PGF_{2\alpha}$ was determined by concentration–response curves. Second, a pharmacological approach was utilized to activate the receptor-Gs-AC-PKA cascade. Finally, the G-protein effectors PTX and CTX were utilized to determine the role of G-proteins in mediating the actions of $PGF_{2\alpha}$. This was performed, since much information in the literature suggested that $PGF_{2\alpha}$ may be exerting its actions through a G-protein *(18–24)*.

As we have previously reported, differential responses to $PGF_{2\alpha}$ were observed in response to different concentrations of $PGF_{2\alpha}$ *(25)*. The response to $PGF_{2\alpha}$ could be either luteolytic or luteotropic, depending on concentration. It was found that a high concentration (1 μ*M*) of $PGF_{2\alpha}$ was required in order for it to exert its luteolytic actions. Interestingly, most other concentrations tested resulted in a luteotropic response. This bimodal nature is somewhat usual for prostaglandin actions *(26–31)* in reproductive tissues *(26,31)* and nonreproductive tissues *(30,31)*. In rat hepatocytes, PGE_2 can act in a glycogenolytic and in a antiglycogenolytic fashion, at concentrations of 10 μ*M* and 1 n*M*, respectively. Presumably, these glycogenolytic and antiglycogenolytic actions are mediated through the inositol triphosphate and cAMP pathways, respectively. Interestingly, $PGF_{2\alpha}$ is known to regulate both of these pathways. In addition to being luteotropic and luteolytic in the presence of hCG, in the absence of gonadotropin, we have also found $PGF_{2\alpha}$ to be trimodal in its effects, being stimulatory, inhibitory, or ineffectual, depending on culture time, concentration, and confounding interactions *(25)*. Moreover, we have found that these effects are independent from the effects in the presence of hCG, such that conditions that permit luteolytic effects in the presence of hCG do not necessarily dictate that this hormone will inhibit steroidogenesis in the absence of hCG, and visa versa. This observation is consistent with the present results in that although the experiments presented here demonstrated $PGF_{2\alpha}$-mediated inhibition of gonadotropin-stimulated steroidogenesis, not all of these experiments had consistent

responses to $PGF_{2\alpha}$ alone. Although, the luteolytic and luteotropic effects of $PGF_{2\alpha}$ are now well defined in the presence of gonadotropin, the variable effects of $PGF_{2\alpha}$ in the absence of gonadotropin still need to be defined. Perhaps the physiological effects of $PGF_{2\alpha}$ are gonadotropin-dependent.

When gonadotropic signal transduction pathway was activated at the receptor, G_s or AC level $PGF_{2\alpha}$ was capable of inhibiting the response, indicating that $PGF_{2\alpha}$ is acting at a level below AC. However, the inability of $PGF_{2\alpha}$ to inhibit this pathway when activated by a high concentration of Db cAMP suggests that $PGF_{2\alpha}$ is acting at a level above the activation of PKA. Thus, it is possible that $PGF_{2\alpha}$ is exerting its antigonadotropic actions through PDE, a suggestion supported by the literature *(1)*. Alternately, another pathway that exerts crosstalk with the cAMP pathway may exist.

It was found that $PGF_{2\alpha}$ is exerting its antigonadotropic actions through a PTX-sensitive G protein. These data were supported by the ability of $PGF_{2\alpha}$ to inhibit CTX-stimulated progesterone and estradiol (not shown) production, and the reversal of this inhibition by the addition of PTX. In the sheep, $PGF_{2\alpha}$ has been shown to inhibit LH-stimulated cAMP levels via activation of PLC with a PTX-sensitive G-protein, although, PTX did not lead to a reduction in $PGF_{2\alpha}$-stimulated hydrolysis of PIP_3 *(47)*. These results taken with the present findings support the suggestion that $PGF_{2\alpha}$ is acting through a PTX-sensitive G protein on the cAMP pathway. Although the present results indicate that the antigonadotropic effects of $PGF_{2\alpha}$ are exerted through a PTX-sensitive G protein, it is unclear if they are mediated through $G_{\alpha i}$, $G_{\alpha p}$, $G_{\beta\gamma}$, or a combination of these subunits.

Reports in the literature support the role of G proteins in the signal transduction of $PGF_{2\alpha}$. As mentioned above, hGLCs have been examined immunocytochemically to reveal a number of G-protein α-subunits, including: $G_{\alpha s}$, $G_{\alpha i3}$, $G_{\alpha i1,2}$, and $G_{\alpha p}$ (namely, $G_{\alpha q}$ and $G_{\alpha 11}$) *(24)*. Intracellular cAMP levels in hGLCs appear to be regulated by the ratio of $G_{\alpha s}$ and $G_{\alpha i}$-subunits, whereas $G_{\alpha q,11}$ and $G_{\alpha i}$ levels regulate the accumulation of inositol phosphates *(24)*. Further supporting a role for G proteins in the signal transduction of $PGF_{2\alpha}$ are the sequences and predicted structure of the cloned prostanoid receptors. All of these receptors possess the seven-transmembrane domain structure characteristic of G-protein-coupled receptors *(18–23)*. Additionally, the human EP_3-family of receptors are capable of inhibiting cAMP production through a PTX-sensitive G protein *(32)*. It is not known if $PGF_{2\alpha}$ is acting through a single or multiple G-proteins, as is seen in the actions of gonadotropin-releasing hormone (GnRH) in the gonadotrope *(33)*.

Previous studies have shown that $PGF_{2\alpha}$ is capable of inhibiting LH-, CTX-, forskolin- and Dbc AMP- (in contrast to this study) stimulated progesterone production in bovine luteal tissues *(34)*. This inhibition is likely owing to a $PGF_{2\alpha}$-mediated reduction in both cAMP accumulation and sensitivity to cAMP *(35,36)*. $PGF_{2\alpha}$ is known to reduce AC activity and increase PDE activity *(37)* through PKC *(38)*, thus, explaining the reduced cAMP accumulation owing to $PGF_{2\alpha}$. As one would expect, the PDE inhibitors 3-isobutyl-1-methylxanthine and Ro-20-1724 block $PGF_{2\alpha}$-mediated inhibition of cAMP *(39)*. The present findings support the suggestion that PDE is mediating the luteolytic effects of $PGF_{2\alpha}$, since they demonstrate that $PGF_{2\alpha}$ is likely acting at a level below the generation of cAMP, but above the activation of PKA. As the cAMP signal transduction system is currently understood, the only step "below" the generation of cAMP by AC and above the activation of PKA is PDE.

Mammalian granulosa or luteal cells treated with $PGF_{2\alpha}$ demonstrate elevated phospholipase C activity and an increase in its downstream products *(9–13,40,41)*. It has been suggested that $PGF_{2\alpha}$ is inhibiting cAMP and progesterone production via this rise in inositol phosphates and/or calcium *(42,43)*, although a direct link between these two pathways has not been clearly established. Numerous reports have demonstrated $PGF_{2\alpha}$-mediated luteolysis in the presence of inositol phosphate, calcium, and calmodulin inhibitors *(14–17)*. Further confusing the issue, there are reports of PLC products stimulating progesterone production, and the fact that LH can stimulate *(11)*, and has been shown even to potentiate $PGF_{2\alpha}$-stimulated IP_3 production *(11)*. Nonetheless, there still exists the possibility that the IP_3 and calcium pathways are coupled indirectly to cAMP degradation through PDE. This suggestion needs to be investigated.

In conclusion, $PGF_{2\alpha}$ appears to be a bimodal regulator of steroidogenesis in the human ovarian cell, such that it can mediate both a luteotropic and a antigonadotropic response. Additionally, through a PTX-sensitive G protein, $PGF_{2\alpha}$ inhibits progesterone production at a level below AC, and above the activation of PKA. These results are important in understanding the mechanism of action of $PGF_{2\alpha}$ and further support the role of this hormone as local regulator of ovarian function.

Materials and Methods

GLC Collection and Culture

The use of hGLC was approved by the Clinical Screening Committee for Research and Other Studies Involving Human Subjects of the University of British Columbia. GLCs were harvested in conjunction with the oocyte collection in the University of British Columbia's in vitro fertilization program. Throughout the precollection period, follicular development was monitored using estradiol assays and ultrasonography. Following pituitary downregulation with a GnRH analog (Synarel, Syntex; Montreal, Quebec) and when estradiol levels were <150 pmol/L, follicular development was stimulated with human menopausal gonadotropin (hMG) (Humegon 75 IU

FSH and 75 IU LH, Organon, Scarborough, Ontario; or Fertinorm 75 IU FSH, Serono, Oakville, Ontario). When three or more follicles reached a diameter >16–18 mm and estradiol levels were >5–7000 pmol/L, final maturation was induced with hCG (10,000 IU; Serono). Thirty-two to 36 h later, follicles were harvested using a transvaginal approach. GLCs were harvested from the follicular fluid following oocyte identification and removal. Multiple follicles from each patient were pooled in order to obtain enough cells for the number of treatments performed. Only follicles with a diameter >12 mm were used.

Following centrifugation (1000*g*) of the follicular contents, the supernatant was decanted, and cells were resuspended in medium 199 (M199; Gibco BRL Life Technologies, Burlington, Canada), supplemented with 10% fetal bovine serum (FBS, Gibco). The resuspended cells were layered on top of a 40% Percoll gradient (Sigma, St. Louis, MO) in M199. The gradient was centrifuged (1700*g*; 10 min; 22°C), in order to remove red blood cells. Following collection from the M199/Percoll interphase, granulosa cells were washed and resuspended (10^5 cells/0.5 mL) in M199, supplemented with 10% FBS, sodium penicillin (100 IU/mL; Gibco) and streptomycin (100 µg/mL; Gibco), and plated on 48-well plates (Corning, NY; 0.5 mL cell suspension/well). A second density gradient was performed if red blood cells accounted for more than 1% of the total cell numbers in the final preparation (as determined by hemocytometry). In preliminary experiments, the luteolytic and luteotropic responses to $PGF_{2\alpha}$ were found to be at their greatest in cells cultured for 8 d (as compared to 1-, 4-, and 14-d precultured cells). Thus, the underlying studies utilized 8-d (d 8) precultured cells, which had media changed every 2–3 d throughout the culture period.

The concentration of $PGF_{2\alpha}$ utilized in these studies was determined by a concentration–response curve, which suggested that 1 µ*M* was required for an optimal antigonadotropic response (Fig. 1). Following the below-described treatments, media were removed and stored at –20°C until assayed for progesterone concentrations. All treatment media were made up in serum-free M199 (supplemented with androstenedione at 5×10^{-7} *M*). Drugs and hormones used in these experiments were purchased from Sigma Chemical Co.

In the first study, stepwise activation of the gonadotropic signal transduction pathway was performed by incubating cells (24 h) with hCG (1 IU/mL), Iso (10^{-5} *M*), CTX (1 µg/mL), forskolin (10^{-5} *M*), and Db cAMP (10^{-5} *M*), in the absence or presence of $PGF_{2\alpha}$ [10^{-6} *M*].

Cells used in PTX/CTX studies were pretreated (18 h) with M199 supplemented with vehicle, PTX (50 ng/mL), CTX (1 µg/mL), or PTX plus CTX. Pretreatment was performed in order to ensure complete inhibition (PTX) or activation (CTX) of the G-proteins prior to the start of the experiment. Following the pretreatment period, cells were exposed to M199-containing vehicle, PTX, CTX, or PTX plus CTX, plus either vehicle, hCG (1 IU/mL), $PGF_{2\alpha}$ (10^{-6} *M*), or hCG plus $PGF_{2\alpha}$ (for 24 h).

Radioimmunoassay (RIA) of Progesterone and Estradiol

The progesterone concentrations in culture media were determined by specific RIAs, as previously described *(44,45)*, with the following modification: phosphate-buffered saline was replaced by a phosphate buffer, containing Na_2HPO_4 (0.04 *M*) and NaH_2PO_4 (0.04 *M*), at pH 7.4.

DNA Quantification

DNA quantification was performed using a modified version of Mates method *(46)*. Briefly, following the treatment period, media were removed and replaced with DNase free tryspin (50 µg/mL; Sigma), in a final volume of 500 µL in phosphate buffered saline (PBS). The plate was stored frozen at (–70°C) until assayed for DNA. At the time of assay, the plate was thawed at room temperature and incubated for 30 min, to allow the trypsin to lyse the cells. During this incubation, pre-prepared Hoechst dye (Bisbenzimide; 20 µg/mL in H_2O; Sigma) was thawed (from –20°C) and diluted (10X in PBS). Following the incubation period Hoechst dye solution was added to each well (at 500 µL/well), mixed and incubated for 5 min, before well DNA levels were determined by spectrofluorometry. Excitation and emission wavelengths were 354 and 458 n*M*, respectively. DNA was quantified by extrapolation from known standards (calf thymus DNA; Sigma), which were prepared by serial dilution (in PBS) over a range of 2.5 to 1000 ng/mL. Standards (1 mL) contained Hoechst dye diluted in similar fashion to samples above. Standards were measured in triplicate.

Analysis of Results

Graph bars show the mean ± SEM of standard mean scores from separate experiments performed on cells from different patients. Standard mean score (z = [value – mean] ÷ SD) standardization of the data was utilized to correct for patient based differences in response magnitude and basal levels. Thus, these data should only be utilized in assessing rank order and relative effects of the treatments; no assessment of response magnitude should be inferred from the data. Statistical differences were determined by ANOVA. Following ANOVA Fisher and Scheffe post-hoc tests were performed on experiments with $n \leq 5$ and $n \geq 6$, respectively, in order to account for replicate number-based differences in the ratio of type I to type II statistical error, the rational being that the conservative Scheffe post-hoc test is too stringent for experiments with a low "n," but will accurately assess individual differences when the "n" is high enough. Different characters above graph bars signify statistical difference (95% confidence interval).

Acknowledgments

The authors wish to express their gratitude to the staff of the UBC IVF lab, for the provision of hGLCs. Additionally, the suggestions and constructive criticism provided by J. Pinter, X. Lin, and D. Stanislaus are gratefully acknowledged. This research was supported by the MRC of Canada. This work was supported by a grant from the Medical Research Council of Canada (to P. C. K. L.). P. C. K. L. is a Career Investigator of the British Columbia Research Institute for Child and Family Health.

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