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ORIGINAL PAPER

Inhibition of rat granulosa cell differentiation by overexpression of Gαq

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Abstract Activation of FSH and LH receptors in undifferentiated granulosa cells (i.e., no prior exposure to FSH) results in comparable induction of progesterone production, but activation of the LH receptor is less effective than FSH in inducing aromatase and the native LH receptor. Because the LH receptor can also activate the Gαq signaling pathway, we investigated whether activation of this pathway could be responsible for these differences. Overexpression of Gαq inhibited FSH induction of both the estradiol and progesterone biosynthetic pathways as well as mRNA levels for cholesterol side-chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), LH receptor (LHr), and P450aromatase (aromatase). This suppression was associated with a reduction ($P < 0.05$) in FSH-stimulated cAMP production. Lower cAMP levels were not due to reduced FSH receptor (FSHr) mRNA levels or reduced levels of Gαs. Phosphodiesterase (PDE) activity and regulator of G-protein signaling 2 (RGS2) mRNA levels were significantly ($P < 0.05$) increased by Gαq, both of which could account for diminished cAMP levels. We conclude that Gαq signaling pathway inhibits both estradiol and progesterone production comparably and thus activation of this pathway does not seem to account for differences between FSH and LH in the regulation of aromatase and the LH receptor.

Keywords Granulosa cells · Gαq signaling · FSH · LH · RGS2

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Introduction

The initiation of preovulatory follicle growth by FSH is associated with the induction of aromatase and the induction of the LH receptor on granulosa cells [1, 2]. Although it is well established that cAMP mediates the actions of FSH on granulosa cells [3], it is uncertain if activation of the cAMP signaling pathway alone is sufficient to account for the stimulatory actions of FSH. In this regard, previous studies demonstrated that FSH is more effective than the cAMP agonist 8Br-cAMP in inducing LHr mRNA in granulosa cells [4]. In addition, we demonstrated recently that the expression of a constitutively active LHr in undifferentiated granulosa cells by adenovirus vectors led to a similar induction of the progesterone biosynthetic pathway compared with FSH, but was less effective than FSH in inducing aromatase and the LHr, despite the fact that comparable amounts of cAMP were produced in response to FSH and LHr stimulation [5].

Collectively, these observations indicate that activation of the FSH receptor (FSHr) is more effective than activation of the LH receptor in inducing the two hallmark genes associated with granulosa cell differentiation, aromatase, and the LH receptor. The preferential actions of FSH on the induction of aromatase and the LH receptor could be due to the activation of other signaling pathways by FSH. In addition to stimulating cAMP production, FSH also stimulates the MAP kinase pathway [6] and protein kinase B [7], and overexpression of constitutively active PKB in granulosa cells enhances the ability of the cAMP pathway to induce aromatase and the LHr [8].

It is also possible that the expression of constitutively active LHr in granulosa cells does not stimulate the induction of aromatase and the native LHr to the same extent as FSH is because activation of the LHr, in addition

to activating the Gαs pathway, may also stimulate other pathways that could suppress the expression of aromatase and the native LHr. In this regard, it has been shown that, in addition to stimulation of the Gαs/cAMP signaling system, activation of the LHr also stimulates the Gαq pathway to increase phospholipase C and protein kinase C (PKC) [9–13]. Because activation of the PKC pathway antagonizes the effects of FSH in granulosa cells [14], it is possible that activation of the LH receptor, by way of its additional activation of Gαq, would not stimulate aromatase and the LHr to the same extent as does FSH. In support of this hypothesis, Donadeu and Ascoli recently demonstrated that overexpression of constitutively active Gαq suppressed the induction of aromatase in granulosa cells by FSH [15].

However, while stimulation of granulosa cells by the constitutively active LHr resulted in lower levels of mRNA for aromatase and the LHr than did FSH stimulation, comparable levels of progesterone production and mRNA levels for P450scc and 3β-HSD were observed in response to both stimuli [5], indicating that stimulation of granulosa cells by the constitutively active LHr selectively reduced the expression of aromatase and LHr. If stimulation of the Gαq signaling system by LHr was responsible for diminished induction of aromatase and the LHr, we would predict that expression of constitutively active Gαq in granulosa cells would not interfere with the induction of the progesterone biosynthetic pathway by FSH. Accordingly, we initiated the current study to determine whether activation of the Gαq signaling pathway selectively interferes with the induction of aromatase and the LHr. For this purpose, we examined the effects of expressing constitutively active Gαq in undifferentiated granulosa cells on the ability of FSH to stimulate both the estrogen and progesterone biosynthetic pathways.

Results

Effects of overexpression of constitutively active Gαq on FSH-stimulated estradiol and progesterone production by undifferentiated granulosa cells

Undifferentiated rat granulosa cells were infected with increasing amounts of the adenovirus (Ad-Gαq) [15] that directs the expression of human constitutively active Gαq (GqQ209L) [16]. Twenty-four hours after viral infection, cells were treated with or without FSH (100 ng/ml) and testosterone (30 ng/ml). The culture medium was collected 48 h after FSH stimulation and was analyzed for estradiol and progesterone content by RIA. Figure 1a shows that FSH treatment resulted in a stimulation of estradiol production. This stimulation was reduced in cells infected with increasing amount of Ad-Gαq. Estradiol production was significantly reduced at concentrations higher than 6.25×10^9 viral particles (vp)/ml ($P < 0.05$). A similar effect was observed in progesterone production (Fig. 1b). As a control, undifferentiated granulosa cells were infected with increasing amounts of the adenovirus that directs the expression of β-galactosidase (Ad-β gal), which did not significantly reduce ($P > 0.05$) progesterone or estradiol production at any viral concentration.

Effects of overexpression of Gαq on estradiol and progesterone production by undifferentiated granulosa cells stimulated with increasing FSH concentrations

To determine whether the effects of Ad-Gαq on estradiol and progesterone production are influenced by the magnitude of FSH stimulation, a dose–response study for FSH was conducted in primary cultures of undifferentiated granulosa cells. Figure 2a shows estradiol production in response to FSH by uninfected undifferentiated granulosa cells (no virus) or by cells infected with Ad-β gal or Ad-Gαq, using a viral concentration of 2.5×10^{10} vp/ml. Concentrations of FSH from 5 to 25 ng/ml produced progressive increases in estradiol production in uninfected cells, whereas at concentrations over 25 ng/ml no further increase was observed. Cells infected with Ad-β gal showed similar response. Infection with Ad-Gαq significantly reduced ($P < 0.05$) estradiol production at concentrations from 25 to 100 ng/ml of FSH when compared to either uninfected cells or cells infected with Ad-β gal. A similar inhibitory effect was observed on progesterone production by cells infected with Ad-Gαq (Fig. 2b) at concentrations from 25 to 100 ng/ml of FSH ($P < 0.05$).

Effects of overexpression of Gαq on FSH-induced mRNAs associated with estradiol and progesterone biosynthetic pathway and granulosa cells differentiations

We performed a ribonuclease protection assay to further compare the effects of Ad-Gαq on the induction of mRNAs for the estrogen and progesterone biosynthetic pathways (Fig. 3a). Undifferentiated granulosa cells infected with Ad-β gal and stimulated with 100 ng/ml of FSH showed an increase in P450scc, 3β-HSD, aromatase, and LHr mRNA levels. As reported previously [15], undifferentiated granulosa cells infected with Ad-Gαq showed a reduction in mRNA levels of FSH-stimulated aromatase. In addition, overexpression of Gαq also reduced the FSH stimulation of mRNAs for P450scc and 3β-HSD, both of which are involved in the progesterone biosynthetic pathway and mRNA for the LHr was reduced, as compared with cells infected with Ad-β gal. Figure 3b illustrates the

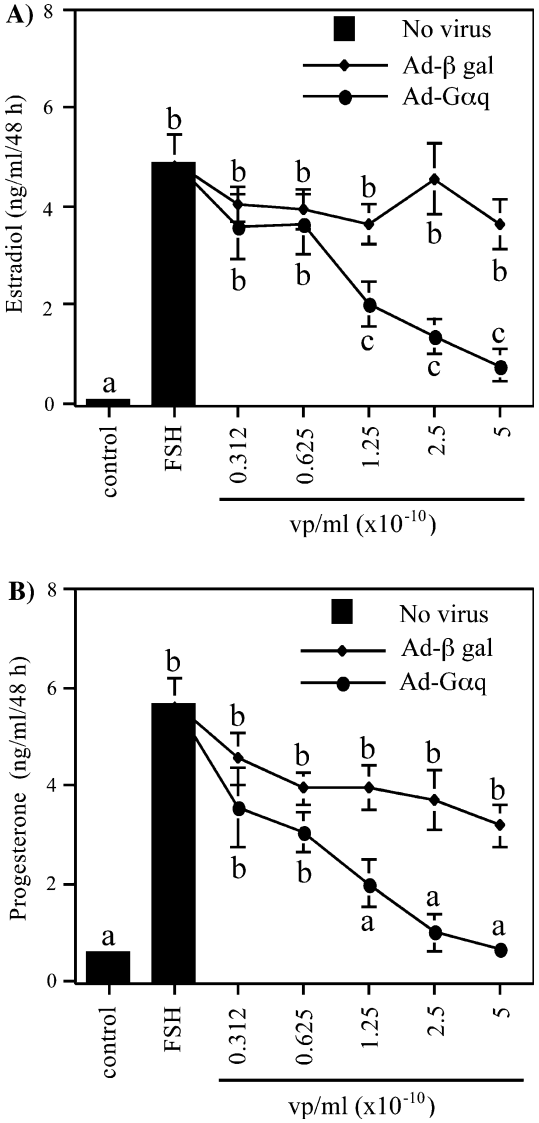

Fig. 1 Effects of expression of Ad-Gαq on estradiol and progesterone production by undifferentiated granulosa cells. Undifferentiated rat granulosa cells were plated overnight in M199 containing 10% FBS. The next morning media and unattached cells were removed and monolayers were exposed to Ad-β gal or Ad-Gαq at concentrations ranging from 0.312 to 5 $\times$ 10^{10} viral particles/ml (vp/ml) for 2 h, after which the virus-containing medium was removed and replaced with M199. After 24 h, medium was removed and replaced with fresh medium containing 30 ng/ml testosterone (control) with or without FSH (100 ng/ml). Forty-eight hours later, medium was collected and analyzed for estradiol (**a**) and progesterone (**b**) content by RIA. Results show mean $\pm$1 SEM of six groups of granulosa cells. Significant differences were considered when $P < 0.05$ and are indicated by different letters in the figure

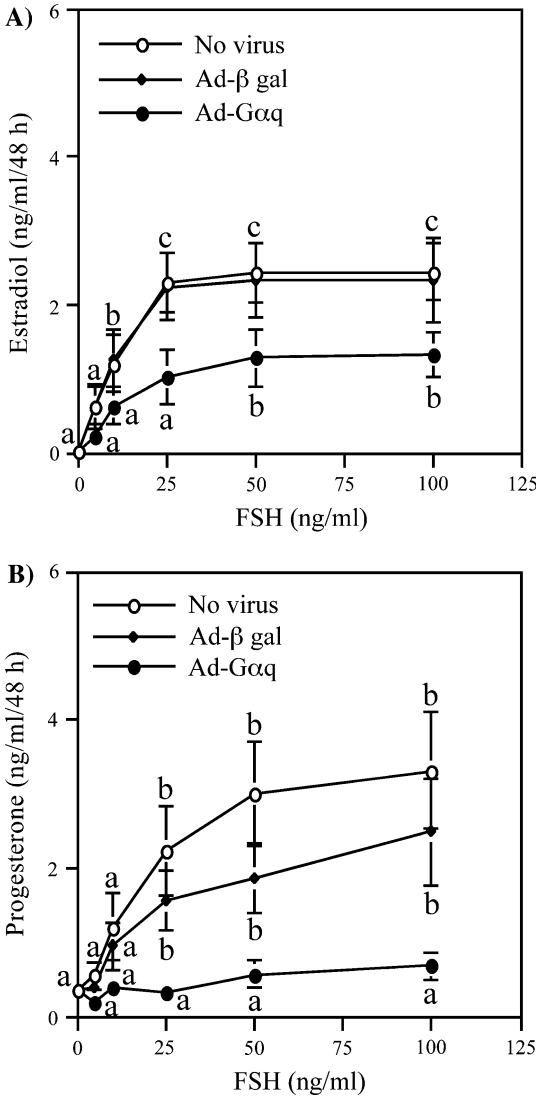

Fig. 2 Effects of Ad-Gαq in the FSH dose–response on estradiol and progesterone production by undifferentiated granulosa cells. Undifferentiated rat granulosa cells were plated overnight in M199 containing 10% FBS. The next morning media and unattached cells were removed and monolayers were exposed to Ad-β gal or Ad-Gαq at a concentration of 2.5 $\times$ 10^{10} viral particles/ml for 2 h, after which the virus-containing medium was removed and replaced with M199. A control group of uninfected cells was included. After 24 h, medium was removed and replaced with fresh medium containing 30 ng/ml testosterone with FSH at concentration of 0, 25, 50, 75, and 100 ng/ml. Forty-eight hours later, medium was collected and analyzed for estradiol (**a**) and progesterone (**b**) content by RIA. Results show mean $\pm$ 1 SEM of six groups of granulosa cells. Significant differences were considered when $P < 0.05$ and are indicated by different letters in the figure

quantitative densitometric analysis of the results from the ribonuclease protection assays and shows that overexpression of Gαq significantly reduced ($P < 0.05$) mRNAs for aromatase, P450scc, 3β-HSD, and LHr in response to FSH stimulation.

Effects of overexpression of Gαq on FSH-stimulated cAMP production by undifferentiated granulosa cells

Our results indicate that overexpression of Gαq reduced both estradiol and progesterone production. Because both depend upon cAMP signaling activated by FSH, we

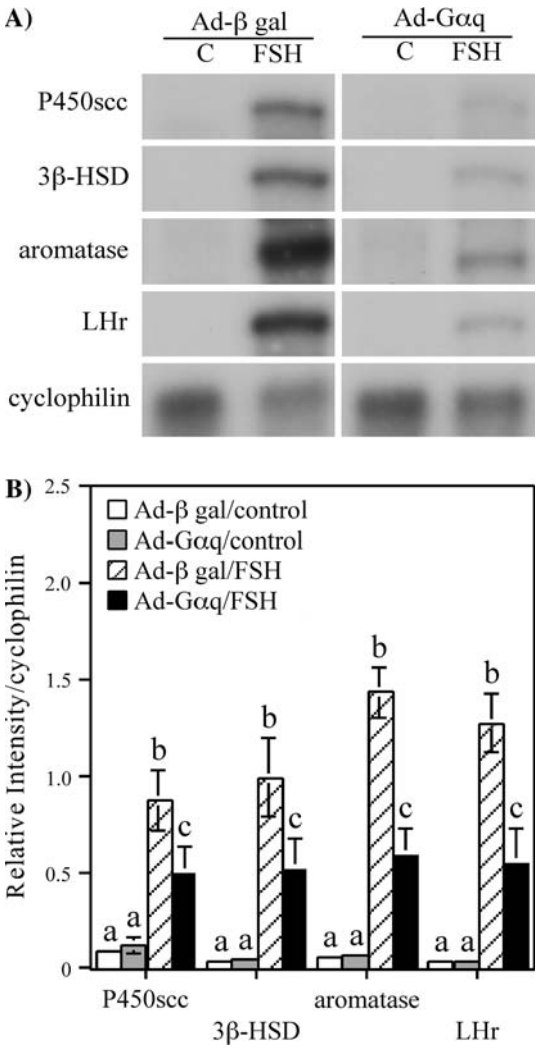

Fig. 3 Effect of Gαq overexpression on FSH-induced mRNAs associated with estradiol and progesterone biosynthetic pathways in granulosa cell. Undifferentiated rat granulosa cells were plated overnight in M199 containing 10% FBS. The next morning media and unattached cells were removed and monolayers were exposed to Ad-β gal or Ad-Gαq at a concentration of 2.5×10^{10} viral particles/ml for 2 h, after which the virus-containing medium was removed and replaced with M199. After 24 h, medium was removed and replaced with fresh medium containing 30 ng/ml testosterone (control, C) with or without FSH (100 ng/ml). Forty-eight hours later, total RNA was extracted from monolayers and analyzed for mRNA by ribonuclease protection assay. The housekeeping gene cyclophilin was included as a loading control. Results shown are representative of six separate groups of granulosa cells (**a**). Densitometry analysis of ribonuclease protection assay was performed using the NIH Image software (**b**), the signal of each mRNA was normalized to the housekeeping gene cyclophilin (relative intensity). Results show the mean ± 1 SEM of six groups of granulosa cells. Significant differences were considered when $P < 0.05$ and are indicated by different letters in the figure

investigated whether FSH-stimulated cAMP production is also affected by overexpression of Gαq. Figure 4a shows that FSH treatment (100 ng/ml) resulted in stimulation of cAMP production ($P < 0.05$) in undifferentiated granulosa cells (no virus). This stimulation was reduced in cells infected with increasing amount of Ad-Gαq, and this reduction was significant ($P < 0.05$) at concentrations of virus greater than 1.25×10^{10} vp/ml. Undifferentiated granulosa cells infected with increasing amounts of Ad-β gal did not exhibit reduced cAMP production at any viral concentration ($P > 0.05$). Figure 4b shows FSH-stimulated cAMP production by undifferentiated granulosa cells infected with a constant amount of either Ad-β gal or Ad-Gαq, and increasing amounts of FSH. Concentrations of FSH from 5 to 100 ng/ml produced progressive increases in cAMP production in granulosa cells in the absence of added viral vectors. Infection with Ad-Gαq reduced FSH-stimulated cAMP production ($P < 0.05$) at concentrations of FSH from 50 to 100 ng/ml when compared to either uninfected granulosa cells or granulosa cells infected with a comparable amount of the control Ad-β gal viral vector.

Effects of overexpression of Gαq on estradiol and progesterone production by undifferentiated granulosa cells stimulated with increasing 8Br-cAMP concentrations

The previous study indicated that undifferentiated granulosa cells infected with Ad-Gαq exhibit a reduction of FSH-stimulated cAMP production. To determine if the reduction of estradiol and progesterone production are the result of the reduction of cAMP levels, a dose–response experiment was performed using 8Br-cAMP in undifferentiated granulosa cells infected with Ad-Gαq or Ad-β gal. Figure 5a and b show that estradiol and progesterone production in response to 8Br-cAMP is virtually identical regardless of whether the cells were infected with Ad-β gal or Ad-Gαq. These findings suggest that the reduction in cAMP levels in cells infected by Ad-Gαq is likely the primary cause for the suppression in estradiol and progesterone production.

Effects of overexpression of Gαq on cAMP production by undifferentiated granulosa cells stimulated with increasing forskolin concentrations

To determine if the direct activation of adenylyl cyclase is affected by Gαq, a dose–response experiment was performed using the adenylyl cyclase activator forskolin (FSK) in granulosa cells infected with Ad-Gαq or Ad-β gal. As shown in Fig. 6, concentrations of FSK from 0.5 to 10.0 μM produced progressive increments in cAMP production in undifferentiated granulosa cells infected with Ad-β gal. Undifferentiated granulosa cells infected with Ad-Gαq showed similar increment in cAMP production with no significant difference ($P > 0.05$), indicating that overexpression of Gαq does not directly inhibit adenylyl cyclase activity. Estradiol and progesterone production induced by FSK were similar between granulosa cells

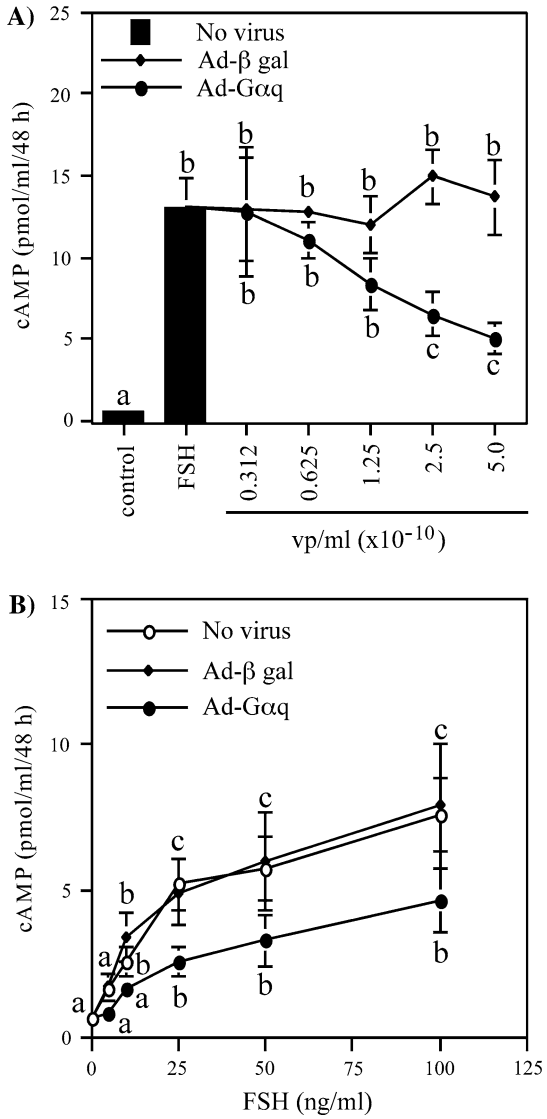

Fig. 4 Effects of expression of Ad-Gαq on cAMP production by undifferentiated granulosa cells. Undifferentiated rat granulosa cells were plated overnight in M199 containing 10% FBS. The next morning media and unattached cells were removed and monolayers were exposed to Ad-β gal or Ad-Gαq at concentrations ranging from 0.312 to 5 $\times$ 10^{10} viral particles/ml (vp/ml) for 2 h, after which the virus-containing medium was removed and replaced with M199. After 24 h, medium was removed and replaced with fresh medium containing 30 ng/ml testosterone (control) with or without FSH (100 ng/ml). Forty-eight hours later, medium was collected and analyzed for cAMP content by RIA (**a**). Undifferentiated rat granulosa cells were plated overnight in M199 containing 10% FBS. The next morning media and unattached cells were removed and monolayers were exposed to Ad-β gal or Ad-Gαq at a concentration of 2.5 $\times$ 10^{10} viral particles/ml for 2 h, after which the virus-containing medium was removed and replaced with M199. A control of uninfected cells was included. After 24 h, medium was removed and replaced with fresh medium containing 30 ng/ml testosterone with FSH at concentrations of 0, 25, 50, 75, and 100 ng/ml. Forty-eight hours later, medium was collected and analyzed for cAMP content by RIA (**b**). Results show mean $\pm$ 1 SEM of six groups of granulosa cells. Significant differences were considered when $P < 0.05$ and are indicated by different letters in the figure

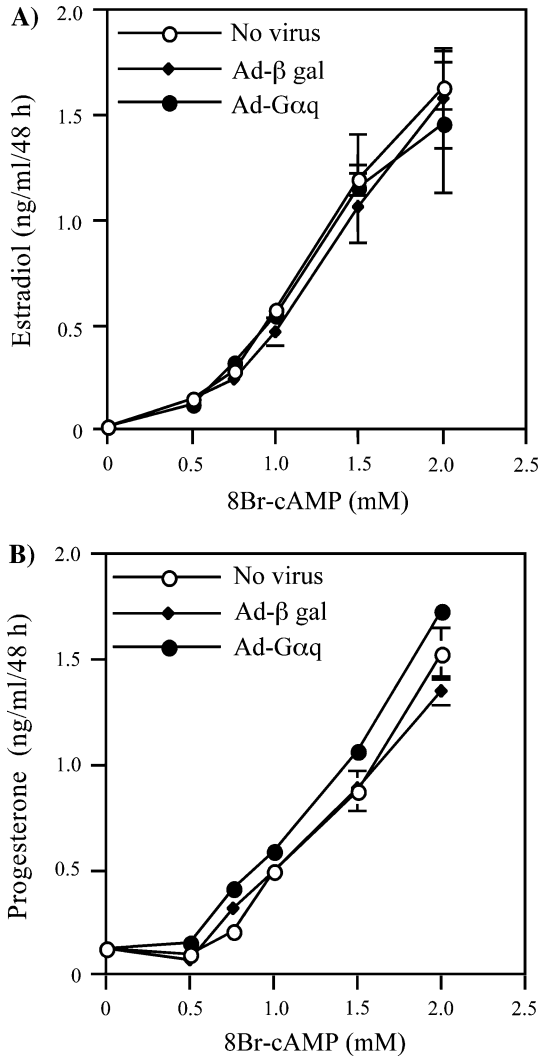

Fig. 5 Effects of expression of Ad-Gαq on estradiol and progesterone production by undifferentiated granulosa cells stimulated with increasing 8Br-cAMP concentrations. Undifferentiated rat granulosa cells were plated overnight in M199 containing 10% FBS. The next morning media and unattached cells were removed and monolayers were exposed to Ad-β gal or Ad-Gαq at a concentration of 2.5 $\times$ 10^{10} viral particles/ml for 2 h, after which the virus-containing medium was removed and replaced with M199. A control of uninfected cells was included. After 24 h, medium was removed and replaced with fresh medium containing 30 ng/ml testosterone with 8Br-cAMP at concentrations of 0, 0.5, 0.75, 1.9, 1.5, and 2.0 mM. Forty-eight hours later, medium was collected and analyzed for estradiol (**a**) and progesterone (**b**) content by RIA. Results show mean $\pm$ 1 SEM of three groups of granulosa cells. Significant differences were considered when $P < 0.05$ and are indicated by different letters in the figure

infected with Ad-β gal and cells infected with Ad-Gαq (data not shown).

Effects of overexpression of Gαq on PDE activity of undifferentiated granulosa cells stimulated with FSH

We investigated whether Gαq decreases cAMP levels in granulosa cells by increasing PDE activity. Figure 7 shows

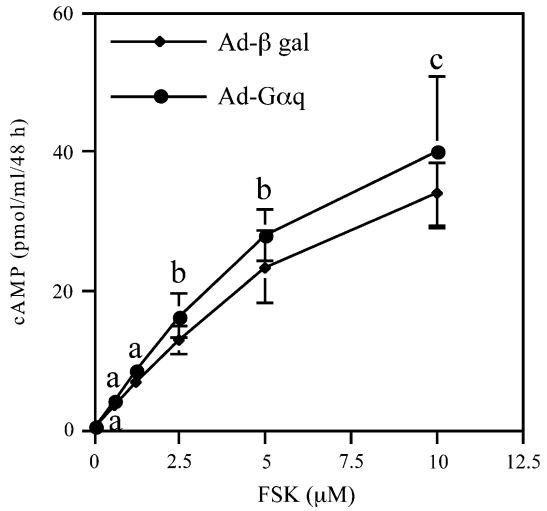

Fig. 6 Effects of expression of Ad-Gαq on cAMP production by undifferentiated granulosa cells stimulated with increasing Forskolin concentrations. Undifferentiated rat granulosa cells were plated overnight in M199 containing 10% FBS. The next morning media and unattached cells were removed and monolayers were exposed to Ad-β gal or Ad-Gαq at a concentration of 5×10^{10} viral particles/ml for 2 h, after which the virus-containing medium was removed and replaced with M199. After 24 h, medium was removed and replaced with fresh medium containing 30 ng/ml testosterone (control) with FSK at concentrations of 0, 0.5, 1.0, 2.5, 5.0, and 100 μM. Forty-eight hours later, medium was collected and analyzed for cAMP content by RIA. Results show mean of ±1 SEM of four groups of granulosa cells

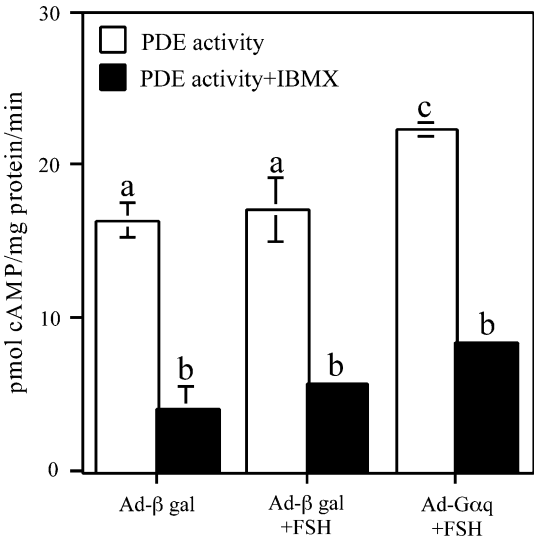

Fig. 7 Effects of expression of Ad-Gαq on PDE activity of undifferentiated granulosa cells stimulated with FSH. Undifferentiated rat granulosa cells were plated overnight in M199 containing 10% FBS. The next morning media and unattached cells were removed and monolayers were exposed to Ad-β gal or Ad-Gαq at a concentration of 5×10^{10} viral particles/ml for 2 h, after which the virus-containing medium was removed and replaced with M199. After 24 h, medium was removed and replaced with fresh medium containing 30 ng/ml testosterone with or without 100 ng/ml of FSH. Forty-eight hours later, cells were collected and whole-cell extracts were prepared. PDE activity was determined using 20 μg of total protein of whole-cell extract in the absence (total PDE activity) or presence of 0.5 mM IBMX, a PDE inhibitor. Results show mean ± 1 SEM of three groups of granulosa cells. Significant differences were considered when $P < 0.05$ and are indicated by different letters in the figure

the total PDE activity in whole-cell extracts from undifferentiated granulosa cells infected with Ad-β gal or Ad-Gαq in the presence and absence of FSH. Undifferentiated granulosa cells infected with Ad-β gal show a similar PDE activity with or without FSH treatment, indicating that FSH did not affect PDE activity. Undifferentiated granulosa cells infected with Ad-Gαq and treated with FSH showed a small but significant ($P < 0.05$) increment in PDE activity when compared to cells infected with Ad-β gal and treated with FSH. In all cases the general PDE inhibitor, IBMX, at 0.5 mM significantly reduced ($P < 0.05$) PDE activity.

Effects of overexpression of Gαq on G-protein levels in undifferentiated granulosa cells

The stimulation of adenylyl cyclase by FSH depends upon the dissociation of Gαs from the heterotrimeric G-protein complex and interaction of the free Gαs subunit with adenylyl cyclase. To determine whether overexpression of Gαq has an affect on the protein levels of the Gαs subunit, whole-cell extracts of granulosa cells from undifferentiated granulosa cells infected with Ad-Gαq or Ad-β gal, untreated or treated with FSH, were analyzed by immunobloting. Figure 8 shows that the protein levels of Gαs (long and short isoforms) and Gβ subunits are unchanged during FSH treatment when compared with the control. Undifferentiated granulosa cells that overexpress Gαq show similar protein levels of Gαs isoforms and Gβ subunits as compared with cells infected with Ad-β gal, indicating that overexpression of Gαq does not diminish cAMP levels by decreasing the expression of Gαs. Gαq levels in whole-cell extracts of granulosa cells infected with Ad-Gαq were compared with those of cells infected with Ad-β gal by inmunoblotting. Results indicated that mean levels of Gαq normalized to GAPDH in cells infected with Ad-Gαq were 1.49 ± 0.19 while normalized Gαq levels in Ad-β gal-infected cells were 1.17 ± 0.15.

Effects of overexpression of Gαq on RGS2 and FSHr mRNA levels in undifferentiated granulosa cells

The family of proteins classified as regulators of G-protein signaling (RGS) modulate the activities of G-proteins [17, 18]. It has been reported that one member of this family, RGS2, is expressed in rat ovaries in response to the ovulatory LH stimulus [19]. To investigate if Gαq affects the expression of RGS2, mRNA levels for RGS2 were analyzed by RT-PCR in granulosa cells infected with Ad-Gαq. Figure 9a shows that RGS2 mRNA levels are increased by

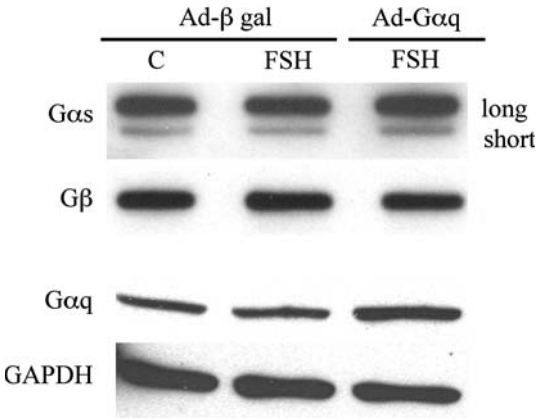

Fig. 8 Effect of overexpression of Gαq on G-protein levels in undifferentiated granulosa cells. Undifferentiated rat granulosa cells were plated overnight in M199 containing 10% FBS. The next morning media and unattached cells were removed and monolayers were exposed to Ad-β gal or Ad-Gαq at a concentration of 5×10^{10} viral particles/ml for 2 h, after which the virus-containing medium was removed and replaced with M199. After 24 h, medium was removed and replaced with fresh medium containing 30 ng/ml testosterone (control, C) with or without FSH at a concentration of 100 ng/ml. Forty-eight hours later, cells were collected and whole-cell extracts were prepared. Western blot analysis of the whole-cell extracts was performed using 10 μg of protein for Gαs or Gβ detection and 40 μg of protein for Gαq detection. The two isoforms of Gαs subunit (long and short) were detected using the ACS antisera; and the Gβ subunit was detected using the BC1 antisera. Gαq protein levels were detected by Western blot using anti-Gαq (E-17). Protein loading was analyzed by using anti-GAPDH (6C5). Results shown are representative of three separate groups of granulosa cells

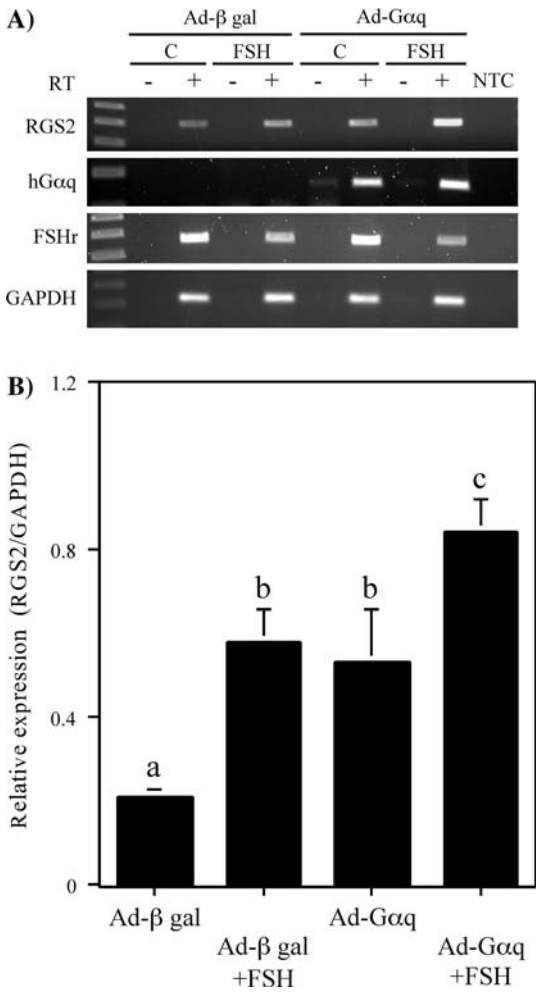

Fig. 9 Effect of overexpression of Gαq on RGS2 and FSHr mRNA levels in undifferentiated granulosa cells. Undifferentiated rat granulosa cells were plated overnight in M199 containing 10% FBS. The next morning media and unattached cells were removed and monolayers were exposed to Ad-β gal or Ad-Gαq at a concentration of 2.5×10^{10} viral particles/ml for 2 h, after which the virus-containing medium was removed and replaced with M199. After 24 h, medium was removed and replaced with fresh medium containing 30 ng/ml testosterone (control, C) with or without FSH at a concentration of 100 ng/ml. Forty-eight hours later, total RNA was extracted from the monolayers and the levels of RGS2, human Gαq, and FSHr mRNA were analyzed by RT-PCR in the presence or absence of the reverse transcriptase (±RT) (**a**). The housekeeping gene GAPDH was included as a loading control and a no-template control (NTC) for PCR was included. Results shown are representative of five separate groups of granulosa cells. Densitometry using the NIH image 1.61 software analyzed the change in the levels of RGS2 mRNA; the signal of RGS2 was normalized to the housekeeping gene GAPDH (relative expression) (**b**). Results show mean ± 1 SEM of five groups of granulosa cells. Significant differences were considered when $P < 0.05$ and are indicated by different letters in the figure

FSH treatment in both granulosa cells infected with Ad-Gαq or Ad-β gal. Moreover, cells infected with Ad-Gαq show higher RGS2 mRNA levels than the cells infected with Ad-β gal in both the presence and absence of FSH. Figure 9a also shows that infection of granulosa cells with Ad-Gαq resulted in increased mRNA levels for the human Gαq recombinant protein demonstrating the effectiveness of the adenovirus vector**.** Additionally, Fig. 9a shows that FSHr mRNA was decreased by 29% following FSH treatment but was not reduced further by Gαq ($P > 0.05$). Figure 9b shows the densitometric analysis of RGS2 mRNA results from the RT-PCR assay. FSH treatment significantly induced ($P < 0.05$) RGS2 mRNA in undifferentiated granulosa cells infected with either Ad-Gαq or Ad-β gal. Overexpression of Gαq significantly increased ($P < 0.05$) RGS2 mRNA levels in the presence and absence FSH as compared with cells infected with Ad-β gal.

Discussion

Our results support previous findings that overexpression of Gαq inhibits FSH-stimulated aromatase activity during granulosa cell differentiation [15]. In addition, our results show that Gαq also inhibits FSH-stimulated progesterone production. The inhibitory effect of Gαq on estrogen and progesterone production correlates with a reduction in the

levels of mRNA levels of the genes associated with these biosynthetic pathways, namely aromatase for estradiol biosynthesis and P450scc and 3β-HSD for progesterone biosynthesis.

The suppressive effect of overexpression of Gαq on estradiol and progesterone seen in our study was likely due to a reduction in FSH-stimulated cAMP production because the permeable cAMP derivate 8Br-cAMP reversed the inhibitory effect of Gαq on both estradiol and progesterone production (Fig. 5). Our observations that cAMP levels were reduced by expression of Gαq differs from that reported previously [15] which showed no change in cAMP production, as assessed by measuring intracellular plus extracellular cAMP content after 4 h of stimulation by FSH [15]. In our study, we chose to measure extracellular cAMP levels after 48 h of stimulation by FSH because it has been previously demonstrated that the induction of LH receptor by FSH requires the continuous presence of FSH over the entire 48 h of culture and during this induction period the pattern of extracellular cAMP paralleled that of the intracellular cAMP [20]. The ability of 8Br-cAMP to override the antagonistic effects of Gαq on estrogen production in our study does not agree with previous work which demonstrated that this cAMP analog failed to reverse the inhibitory effects of Ad-Gαq on FSH-stimulated aromatase mRNA. [15]. Currently, we have no explanation for this difference.

Gαq signaling activates phosphoinositide-specific PLC which in turn hydrolyzes phosphatidylinositol-4,5-bisbiphosphate to produce two second messengers, diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (IP_3); DAG activates PKC while IP_3 releases Ca^{++} from intracellular stores. Previous studies examined the effects of PKC stimulation by phorbol 12-myristate 13-acetate (PMA) on FSH-induced granulosa cell differentiation and found that PMA inhibited both FSH-stimulated LH receptor induction and progesterone production [14], similar to what we observed in the present study. They also found, similar to our study, that cAMP production was reduced approximately 50% by PMA and proposed that the reduced cAMP could be due to a decrease in FSHr and/or an inhibitory effect on receptor coupling to adenylyl cyclase.

In our current study, we did not observe an effect of Ad-Gαq on either mRNA for the FSHr, or on Gαs or Gβ protein levels. Assuming that FSHr mRNA levels parallel those of the actual protein, these findings indicate that the lower cAMP levels were not due to reduced FSHr levels or reduced levels of Gαs. We did, however, observe an approximate 25% increase in PDE activity in granulosa cells infected with Ad-Gαq. However, it is unlikely that the reduction in cAMP levels and the reduction in steroid production we observed were due solely to increased PDE activity because although the PDE inhibitor IBMX was able to inhibit the PDE activity of whole-cell extracts of granulosa cells, it was unable to reverse completely the effect of Ad-Gαq on cAMP levels and the inhibition of estrogen and progesterone production in intact cells (data not shown).

We also observed a significant increase in the level of mRNA for RGS2, a member of the regulator of G-protein signaling family of proteins, in response to Ad-Gαq. Interestingly, it has been demonstrated previously that mRNA for RGS2 is also increased in rat granulosa cells in response to an ovulatory dose of hCG [19]. In addition, RGS2 mRNA and protein levels are elevated in cardiomyocytes by overexpression of Gαq [21] as well as by activation of inositol pathways in astrocytoma cells [22]. RGS proteins may influence G protein signaling at multiple levels including acting as a GTPase activating factor (GAF) of Gα proteins as well as by direct inhibition of effectors of G protein signaling such as adenylyl cyclase [23, 24] and PLC [25]. Although it does not appear that RGS2 has direct GAF activity, recent studies have suggested that RGS2 may directly inhibit adenylyl cyclase [24] and lower cAMP levels in other cell systems, possibly by direct interaction with Gαs [23]. Thus, it is possible that a component of the decrease in FSH-stimulated cAMP accumulation and inhibition of steroid production by Ad-Gαq seen in our current study may be accounted for by increased expression of RGS2 or possibly other members of the RGS family.

It is likely that the Gαq inhibition of cAMP production and subsequent granulosa cell differentiation was mediated by protein kinase C, because as demonstrated previously, overexpression of Gαq led to activation of the PLC pathway [15] and activation of this pathway by phorbol esters inhibits both estradiol and progesterone production by undifferentiated granulosa cells [14]. In addition, overexpression of Gαq increases RGS2 levels in cardiomyocytes [21] and activation of PLC pathway in astrocytoma cells [22] also increases RGS2 levels. However, we were unable to document this directly by using PKC inhibitors because long-term exposure of granulosa cells in culture with GF109203X led to cell death and the Ca^{++} chelator BAPTA could not overcome the effect of Gαq over expression (data not shown). Despite the uncertainty regarding the precise mechanism by which overexpression of Gαq inhibits FSH actions on granulosa cell differentiation, it is clear that Gαq inhibits both estradiol and progesterone production comparably and thus activation of this pathway by LH would not seem to account for differential regulation of the estrogen and progesterone biosynthetic pathways. This conclusion is further strengthened by our previous observations that other stimulators of the cAMP signaling system including

forskolin, 8-Br-cAMP, and a constitutively active Gαs signaling protein, all of which lie distal to the LHr, also fail to induce the expression of aromatase and the LHr to the same extent as does FSH [5, 8].

Materials and methods

Chemical and reagents

Unless otherwise noted, all reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO). Human FSH (AFP-4161-B; IU second IRP FSH per milligram) was generously provided by Dr. A. F. Parlow [National Hormone and Pituitary Program, National Institute of Diabetes and Digestive Kidney Diseases, National Institute of Health (NIH), Torrance, CA].

Adenovirus vectors

An adenovirus vector that directs the expression of human constitutively active Gαq (Ad-Gαq), provided by Dr. Mario Ascoli (The University of Iowa Carver College of Medicine, Iowa City, IO), was used as described previously [15]. The adenovirus vector that directs the expression of β-galactosidase (Ad-β gal) was provided by Dr. Joseph Alcorn (University of Texas-Houston Medical School, Houston, TX). Both viral vectors use a cytomegalovirus promoter. Each adenovirus was amplified in 293T cells, and the virus concentrations were determined by measuring the OD at 260 nm using a ratio of 1.1×10^{12} viral particles per 1 OD unit [26].

Granulosa cell culture

The University of Pittsburgh Institutional Animal Care and Use Committee approved all procedures. Immature female rats (24-day old) were purchased from Hilltop Lab Animals (Scottdale, PA). Granulosa cells were collected from the ovaries by puncturing follicles with a 25-gauge hypodermic needle into Medium 199 (M199; GIBCO-Invitrogen Corp., Grand Island, NY) then centrifuged at 100g for 10 min and resuspended into M199 containing 10% fetal bovine serum (FBS; Atlanta Biologicals, Norcross, GA). Granulosa cells were seeded into 6-well ($\sim 10^6$ cells/well) or 24-well ($\sim 2 \times 10^5$ cells/well) tissue culture plates and allowed to attach overnight. The next morning, medium and unattached cells were removed and the granulosa cell monolayers were exposed to adenoviruses for 2 h. After infection, the virus-containing medium was removed and replaced with fresh serum-free M199 and incubated for 24 h. The next day, cells were exposed to stimulatory agents, as described in the figure legends, and maintained in serum-free M199 containing testosterone at final concentration of 30 ng/ml. At the end of the experiment, tissue culture medium was collected, boiled for 10 min to inactivate phosphodiesterases and stored at −20°C for subsequent RIAs. Where indicated, total RNA was prepared from the cell monolayers using RNA-Bee (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's directions. For descriptive purposes, granulosa cells from sexually immature 24-day-old rats are referred to as undifferentiated because they lack the presence of functional LHr and do not produce estrogen or progesterone under basal conditions. However, these cells respond to FSH with respect to the production of cAMP and induction of LHr and the induction of the estrogen and progesterone biosynthetic pathways [5].

RIA

Estradiol and progesterone content in the culture medium were determined by RIAs as described previously [27]. cAMP concentration in culture medium was analyzed by RIA using [^{125}I] cAMP-2-0′ monosuccinlyl cAMP tyrosine methyl ester [28] and anti-cAMP in accordance with the instructions provided by the National Hormone and Pituitary Program.

mRNA analysis

Samples of total RNA (0.5–1 μg) were analyzed for cholesterol side-chain cleavage enzyme (P450scc), 3β-Hydroxysteroid dehydrogenase (3β-HSD), aromatase (P450aromatase), and LHr expression by RNase protection assay according to the instructions provided by the supplier (Ambion, Inc., Austin, TX). Antisense RNA probes were prepared using [α-^{32}P] CTP (Perkin-Elmer Life Sciences, Boston, MA) from the following cDNA inserts: P450scc (bp 18–816) [29]; 3β-HSD (bp 453–932) [30]; P450arom (bp 1034–1295) [31]; rat LHr (bp 1–622) [32]; and cyclophylin (bp 34–142) [33]. After electrophoresis (5% acrylamide containing 8 M urea), gels were dried and exposed to X-ray film for 16–96 h. Densitometric analysis of protected RNA fragments was performed using NIH Image (version 1.61).

Assay of phosphodiesterase activity

Granulosa cells were plated in 6-well dishes and infected with the adenovirus Ad-β gal or Ad-Gαq using a final concentration of 5×10^{10} vp/ml. Then cells were stimulated with FSH as described in the figure legends. After stimulation, cells were washed twice with PBS, scraped into cold PBS, centrifuged at 14,000g for 5 min, and resuspended into 200 μl of cold IPA buffer (10 mM Na-

phosphate buffer pH 7.5, 50 mM NaF, 150 mM NaCl, 2 mM EDTA, 3 mM benzamidine, 0.1% Triton X-100, 0.5% Igepal, 5 μg/ml Leupetin, and 20 μg/ml pepstatin A) [34, 35]. The samples were sonicated with two 15 s-bursts in an ice bath with a sonic dismembrator (Model 100, Fisher Scientific Company L.L.C, Pittsburgh, PA) at a setting of 2. Protein concentration of the whole-cell extracts was determined using the Bicinchoninic Acid (BCA) protein assay kit (Pierce Biotechnology, Inc, Rockford, IL). Phosphodiesterase (PDE) activity was determined by the 2-step method described previously [34, 35]; 50 μl sample containing 0.02 mg of protein was mixed with 200 μl incubation buffer (44 mM MOPS pH 7.5, 0.88 mM EGTA, 16.6 mM Mg acetate, 220 μg/ml BSA) containing 200 pmol of cAMP and 100,000 cpm of ^{3}H-cAMP (31 Ci/mmol, 1 mCi/ml, Perkin-Elmer Life and Analytical Science, Boston, MA). Similar samples were prepared containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX). The reactions were incubated at 30°C for 10 min then stopped by incubation at 100°C for 1 min. After cooling on ice, 20 μl of 1.25 mg/ml *Crotalus atrox* venom nucleotidase was added and incubated at 30°C for 10 min. At the end of the reaction, 250 μl of low-salt buffer (20 mM Tris–HCl, pH 6.8) was added to the samples, mixed and divided in two aliquots of 250 μl; one for total cpm and the second for the transformed cpm. Ion exchange chromatography was used to separate the produced [2,8-^{3}H]-adenosine, as described previously [36] with few modifications. Briefly, 500 μl of 1:3 slurry of Dowex 1×8 chlorine form anion exchange resin was added to the second aliquot, thoroughly vortexed, equilibrated for 5 min at room temperature, and centrifuged at 1,000*g* for 5 min. The supernatant was removed and radioactivity was counted. For the blank reaction (blank cpm), four tubes containing 50 μl of H_2O instead of whole-cell extract were included in the PDE assay. The phosphodiesterase activity was calculated as describe previously and expressed as pmol cAMP/mg protein/min [37].

Western blot

Granulosa cells were plated in 6-well dishes, infected with the adenovirus and then stimulated with FSH as described above. After stimulation, cells were washed twice with PBS, scraped into cold PBS, centrifuged at 14,000*g* for 5 min, and resuspended into 120 μl TE buffer (50 mM Tris–HCl pH 7.4, and 1 mM EDTA) supplemented with 20 mg/ml phenylmethylsulfonylfluoride, 0.5 mg/ml leupeptin, 200 μM sodium vanadate, and 100 nM microcystin. The samples were sonicated in an ice bath with a sonic dismembrator (Model 100, Fisher Scientific Company L.L.C, Pittsburgh, PA) for 30 s with a setting of 2. Protein concentration was determined by Bradford protein assay (Bio Rad, Life Science Research, Hercules, CA). Whole-cell extracts containing 10 μg of protein for Gαs and Gβ detection or 40 μg of protein for Gαq detection were separated on 12% SDS-PAGE and the resolved proteins were electrophoretically transferred to nitrocellulose membranes. Anti-Gαs western was performed using polyclonal ASC (ASC-B) diluted at 1:2000 in blocking solution which contains 0.5% blocking reagent (Roche Diagnostics Corp, Indianapolis, IN) in TBS (50 mM Tris–Cl pH 7.5, and 150 mM NaCl) and incubated overnight at 4°C [38]. Chemiluminescent detection was accomplished using the BM Chemiluminescence western blotting kit (Roche, Indianapolis, IN) with the anti-rabbit horseradish peroxidase-conjugated secondary antibody diluted to 1:2000 according to the manufacturer's directions. Membranes were stripped and probed with an anti-Gβ using the polyclonal BC1 diluted 1:5000 in blocking solution and incubated for 1.5 h at room temperature [39]. Both antibodies, ASC-B and BC1, were gifts from Dr John D. Hildebrandt (Department of Pharmacology, Medical University of South Carolina, Charleston, SC). Gαq Western blot analysis was performed using the polyclonal anti-Gαq E-17 (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) diluted at 1:500 in blocking solution containing 0.1% Tween and incubated at room temperature overnight. For loading control, membranes were stripped and probed with monoclonal anti-GAPDH 6C5 (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) diluted at 1:2000 and incubated overnight at 4°C. Chemiluminescent detection was accomplished using the BM Chemiluminescence western blotting kit (Roche, Indianapolis, IN) with the anti-mouse horseradish peroxidase-conjugated secondary antibody diluted to 1:4000 according to the manufacturer's directions.

RT-PCR analysis

Total RNA was isolated as above and samples containing 0.5 μg of total RNA were reverse transcribed using random hexamer primers (I.D.T., Coralville, IA). Similar reactions were included for each sample without the reverse transcriptase (RT) to detect genomic DNA contamination in the RNA preparation. The PCRs were performed in a volume of 25 μl containing 1× PCR buffer Absolute (Gene Choice, Frederick, MD), 600 μM dNTPs (Promega, Madison, WI), 70 nM of each forward and reverse primer (as described below), 0.625 U *Taq* DNA polymerase (Gene Choice, Frederick, MD), and 5 μl template. The templates are cDNA obtained from the reverse-transcribed RNA in presence or absence of RT. The PCRs were carried out at 95°C for 5 min, followed by 25–40 cycles at 95°C for 30 s, 60°C, or 62°C or 65°C for 30 s and at 72°C for 1 min, with a final extension step at the end of 72°C for 5 min. After

completion of the PCR amplification, products were analyzed by gel electrophoresis in a 1.5% agarose gel in 1× TBE buffer at 150 V for 30 min. The PCR products were visualized with ethidium bromide staining and pictures were taken. Densitometric analysis of PCR product intensity was performed using NIH Image (version 1.61).

The PCR primers used were the following: to amplify rat RGS2 cDNA, forward 5′-CACCGACTTCATCGAGAA GGA-3′ and reverse 5′-TTTTTCTGGCAGGTTACAGC CT-3′; to amplify human constitutively active Gαq cDNA, forward 5′-TAATGACTTGGACCGCGTAGC-3′ and reverse 5′-TGACCTTAGGCCCCCTACATC-3′; to amplify rat FSHr cDNA, forward 5′-GCTGCCAGCGTCATGGT-ATT-3′ and reverse 5′-TTGGTGTCGCTTGATGAGGA-3′; and to amplify the housekeeping gene rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA forward 5′-GC ATGGCCTTCCGTGTTCCTA-3′ and reverse 5′-GCCAG CCCCAGCATCAAAGGT-3′.

Statistics

Where indicated, results were assessed for statistical significance by ANOVA followed by comparison of group means with Fisher's least significant difference analysis (StatView, version 4.5, Abacus Concepts, Berkeley, CA). In all cases statistical differences were considered at $P < 0.05$.

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