Regulation of ovarian steroidogenesis *in vitro* by gonadotropin in common carp *Cyprinus carpio*: interaction between calcium- and adenylate cyclase-dependent pathways and involvement of ERK signaling cascade

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Abstract

Multiple signal transduction pathways mediating gonadotropin-induced testosterone and 17 β -estradiol (E₂) production were identified in carp ovarian theca and granulosa cells in short-term co-incubation. Inhibitors of voltage-sensitive calcium channels (VSCCs) and calmodulin attenuated human chorionic gonadotropin (HCG)-induced steroid production, whereas modulators of adenylate cyclase and protein kinase A (PKA) increased their production, indicating that both calcium- and PKA-dependent pathways are involved in the regulation of gonadotropin-induced steroidogenesis in carp ovary. Interactions between these two pathways are evident from the positive effect of elevated intracellular calcium on HCG-induced steroid production and the reduction of forskolin (FK)- and dibutyryl cAMP (dbcAMP)-induced steroidogenesis by inhibitors of VSCCs and calmodulin. In this study, we found the involvement of a third signaling pathway, a mitogen-activated protein kinase (MAP kinase), in the regulation of gonadal steroidogenesis in this fish. An antagonist of mitogen-activated protein kinase kinases 1/2 (MEK1/2; also known as MAP2K1/MAP2K2) markedly attenuated HCG-induced steroid production. Cells treated with HCG stimulated MEK1/2-dependent phosphorylation of extracellular signal-regulated protein kinases 1/2 (ERKs1/2) in a concentration and time-dependent manner. Moreover, ERK1/2 activation in cells was mimicked by FK and dbcAMP suggesting that ERK1/2 transduce signal downstream of PKA in HCG-induced ovarian steroidogenesis. Evidence for presence of cross talk between calcium-dependent pathways and this MAP kinase cascade has been shown by demonstrating the inhibitory effects of verapamil and calmodulin on ERK1/2 activation after HCG stimulation. Our results suggest that activation of ERK1/2 by HCG as well as other agents may be a key mechanism for the modulation of gonadotropin-induced steroidogenesis in carp ovary.

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Introduction

The stimulation of gonadal steroidogenesis by pituitary gonadotropins is mediated by various intracellular signaling mechanisms. In ovarian steroidogenic cells, gonadotropins bind to specific membrane G-protein-coupled receptors (GPCRs) and lead to the activation of multiple signal transduction pathways, including the adenylate cyclase/cAMP-dependent protein kinase A (PKA) signaling pathway and calcium/calmodulin-dependent pathways (see review in Leung & Steele (1992) and Van Der Kraak & Wade (1994)). Cross talk among these signal transduction systems has been well documented in many cell types and in response to a variety of receptor agonists (Rasmussen 1981, Bygrave & Roberts 1995, Richards 2001). Moreover, several adenylate cyclase isoenzymes are known to be up- or downregulated by calcium (Guillou *et al.* 1999). Available information also

reported for involvement of mitogen-activated protein kinase (MAP kinase) signaling in the regulation of ovarian steroidogenesis in mammals (Amsterdam *et al.* 2003). The extracellular signal-regulated protein kinases (ERKs) group of MAP kinase includes three kinases (P-42 ERK2, P-44 ERK1, and P-46 ERK1b), which are phosphorylated by the mitogen-activated protein kinase kinases, MEK1 and MEK2 (also known as MAP2K1 and MAP2K2; Seger & Krebs 1995, Lewis *et al.* 1997). In mammalian granulosa cells, ERK1/2 is activated in response to gonadotropins and is important in the regulation of gonadotropin-induced ovarian steroidogenesis (Moore *et al.* 2001, Dewi *et al.* 2002, Amsterdam *et al.* 2003, Su *et al.* 2006, Woods & Johnson 2007). Moreover, occurrence of a cross talk between either adenylate cyclase- or calcium-dependent signaling pathway and MAP kinase cascade has also been demonstrated (Richards 2001; reviewed in Agell *et al.* (2002)).

Previous studies have demonstrated the involvement of adenylate cyclase- and calcium-dependent pathways in mediating the effects of gonadotropins on ovarian steroidogenesis in teleosts (Van Der Kraak & Wade 1994, Benninghoff & Thomas 2005, 2006a). However, an interaction between these pathways in the regulation of ovarian steroidogenesis in fish is not well understood. A recent study showed that testosterone synthesis in Atlantic croaker ovarian follicles induced by activators of adenylate cyclase and PKA is sensitive to antagonists of voltage-sensitive calcium channels (VSCCs), calmodulin, and calcium-/calmodulin-dependent protein kinases (CaMKs), indicating the occurrence of a cross talk between adenylate cyclase- and calcium-dependent pathways (Benninghoff & Thomas 2006a). Although available information indicates the involvement of ERKs in the regulation of gonadotropin-induced steroidogenesis in mammalian granulosa cells, the precise role of this protein kinase in mediating hormone-induced steroidogenesis in fish ovary is unclear. Recently, Benninghoff & Thomas (2006b) suggested an involvement of MAP kinase signaling cascade in gonadotropin-induced steroidogenesis in Atlantic croaker ovarian follicles. Interestingly, they have indicated the occurrence of a cross talk only between adenylate cyclase and MAP kinase pathways in these processes.

Therefore, the primary objective of this study was to investigate whether gonadotropin induces activation of ERK1/2 in mediating gonadotropin-induced steroid production in common carp (*Cyprinus carpio*) ovarian theca and granulosa cells in short-term co-incubation. We also tried to investigate the possible interactions between adenylate cyclase-/calcium-dependent pathway and MAPKs pathways in gonadotropin-induced steroid production in carp ovarian follicles.

Materials and methods

Chemicals

Dibutyl cAMP (dbcAMP), forskolin (FK), verapamil, calcium ionophore A23187, calmodulin antagonist W-5, dimethylsulfoxide (DMSO), percol, BCS, DMEM/nutrient mixture F-12 Ham, collagenase type-I, and nitrobluetetrazolium/5-bromo-4-chloro-3-indoylphosphate were purchased from Sigma Chemical. Human chorionic gonadotropin (HCG) was a gift from National Hormone and Pituitary Program (Torrence, CA, USA). Adenylate cyclase inhibitor, SQ22536 (RBI, Natick, MA, USA), was a gift from Dr Sib Sankar Roy, Molecular Endocrinology Laboratory, Indian Institute of Chemical Biology, 4 Raja S C Mullick Road, Kolkata, West Bengal, India. MEK inhibitor PD98059, mouse monoclonal antiphospho ERK1/2 antibody P-ERK, and the secondary antibody goat anti-mouse

IgG_{2α} were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal P-ERK (P-E-4) antibody was recommended for detection of ERK1 phosphorylation at Tyr-204 and correspondently ERK2 phosphorylation of multiple species. [³H]testosterone (specific activity 95.0 Ci/mmol) and [³H]17β-estradiol (E₂; specific activity 75.0 Ci/mmol) were purchased from Amersham International Plc. Testosterone and E₂ antibodies were gifts from Prof. Gordon Niswender, Colorado State University, Fort Collins, CO, USA. Verapamil and FK stock solutions were prepared in ethanol and stock solutions of W-5, PD98059, and A23187 were prepared in DMSO so that final concentration of the solvents in the incubation media was <0.1%. All other chemicals used were of analytical grade.

Animals and tissue collection

Adult *C. carpio* (300–400 g body weight), collected from a local fish farm in the month of November, were maintained in running tap water in laboratory concrete tanks (300 l capacity) at 23 ± 1 °C. They were fed with commercial fish food (Shalimar Fish Food; Bird and Fish Food Manufacturer, Mumbai, India). During the month of November in the plains of West Bengal, India, ovary of common carp comprises mostly postvitellogenic follicles (0.5–0.7 mm diameter) with oocytes containing centrally located germinal vesicle and lipid droplets in the cytoplasm were found to initiate coalescence. Follicular stages were determined by stripping out few follicles through ovipore followed by examination under microscope after fixing them in clearing solution of acetic acid–ethanol–formalin (1:6:3 v/v) for 12 h. Follicles came out through ovipore after stripping if the fish were in postvitellogenic stage. Fish after screening were deeply anesthetized with MS222 and killed by decapitation at 0800 h in the morning. Ovaries were removed and placed in ice-cold Idler's medium containing streptomycin (100 µg/ml) and penicillin (100 IU/ml) adjusted to pH 7.4 (Mukherjee *et al.* 2006), and postvitellogenic stage of the follicles was confirmed after fixing them in clearing solution followed by examination under microscope. Total number of fish examined for this study was 250.

Tissue preparation

Ovaries were dissected into small pieces in ice-cold Idler's medium and oocytes with follicle layers were separated by repeated pipetting and collected in fresh medium. Postvitellogenic follicles were separated from previtellogenic (0.2–0.3 mm diameter) and vitellogenic (0.3–0.4 mm diameter) follicles by sieving them through stainless steel wire mesh (i.d. 0.5 mm). Follicles thus obtained were initially placed in 50 ml

sterile glass beaker for 2 h that contained 5.0 ml medium. This 2 h preincubation was required to waive the surgical shock (Paul *et al.* 2008).

Detailed methods for isolation of theca and granulosa cells from ovarian follicles have been described in our previous article (Paul *et al.* 2010). In brief, ovarian follicles were digested in 0.1% collagenase type-I for 30 min in a 25 ml glass beaker containing calcium–magnesium-free Idler's medium with continual gentle mixing on a rotating shaker. Theca and granulosa cells were separated independently from the ovarian follicles. They were then separated from other cell types by layering on a single-density percol layer adopting the procedure described by Benninghoff & Thomas (2006a). The final pellet was re-suspended in 1.0 ml culture media and cell density was determined by hemocytometer count. Cell viability, which was >90%, was ascertained by Trypan blue exclusion method.

Incubation of common carp theca and granulosa cells

Before each experiment, theca and granulosa cells were mixed and preincubated for 6 h in a 24-well culture plate in DMEM supplemented with 0.2% BCS–DMEM, streptomycin (100 µg/ml), and penicillin (100 IU/ml). The initial density of theca and granulosa cells in the incubation was 0.9×10^5 and 2.1×10^5 cells per well (500 µl) respectively. After 6 h, BCS–DMEM was replaced by serum-free DMEM and incubated in a metabolic shaker bath at $23 \pm 1^\circ\text{C}$ for different time intervals in a 24-well culture plate containing effectors and inhibitors. Cell incubations were visually inspected periodically during the experiments and finally at the end of the incubations to ensure that hormone treatment did not cause any observable change in cell density or morphology. At the end of each incubation, medium was aspirated, centrifuged (3000 g) for 5 min, and the supernatant was stored at -20°C for steroid assay.

Determination of ERK1/2 phosphorylation

For determination of ERK1/2 phosphorylation, theca and granulosa cell mixtures were preincubated in DMEM for 6 h followed by 2 h incubation in SF-DMEM. SF-DMEM medium was replaced twice to reduce basal ERK1/2 phosphorylation levels. Finally, the cells with SF-DMEM media were incubated for varying times containing (described in 'Results' section) various stimulators and inhibitors. After incubations, media were removed; the cells were rinsed with PBS and then lysed with 500 µl lysis buffer. Details of determination of ERK1/2 phosphorylation have been described in our previous article (Paul *et al.* 2009). Briefly, at the end

of incubation, cells were washed with fresh medium, pooled from duplicate wells, and then lysed with 100 µl ice-cold lysis buffer. Cell lysates were centrifuged at 12 000 g for 5 min at 4°C and supernatant was stored at -20°C until further use. For western blot analysis, supernatant was sonicated for 5 s on ice and protein content was determined according to the method described by Lowry *et al.* (1951). An equal volume of protein (20 µg total protein) was electrophoresed through a 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Fermentas Inc. Life Sciences, Glen Burnie, MD, USA). Membranes were blocked for 1 h in 5% blocking solution (Tris-buffered saline with 0.1% Tween-20 and 5% non-fat milk) followed by incubation with primary antibody for overnight at 4°C . Mouse monoclonal anti-phospho ERK1/2 antibody P-ERK (P-E-4) validated earlier for use with *C. carpio* ovarian follicles (Paul *et al.* 2009) was used at 1:2000 dilutions. Bound primary antibody was visualized using corresponding secondary antibody (goat anti-mouse IgG (1:2000 dilutions)), which was tagged with alkaline phosphatase and was developed with nitrobluetetrazolium/5-bromo-4-chloro-3-indoylphosphate.

Statistical analysis

Data obtained from three replicate incubations of theca and granulosa cells isolated from single donor fish showed a similar tendency and therefore a mean of all the three data was considered as one experiment. All data were expressed as mean $\pm$ S.E.M. of five such experiments taking cells from five donor fish or otherwise mentioned in figure legends. After the test for normality and homogeneity, the significance of treatment effects was determined by one-way ANOVA within and across different effectors. Individual comparisons between treatments were made by adopting Bonferroni's multiple comparison tests using SPSS (Chicago, IL, USA). The level of significance chosen was $P < 0.05$.

Results

Effects of modulators of adenylate cyclase and PKA on steroid production

Follicle cells, after 6 h co-incubation in BSA–DMEM, were treated for 16 h in SF-DMEM with increasing concentrations of HCG (0, 25, 50, 100, or 200 ng/ml), the adenylate cyclase activator FK (0, 0.1, 1.0, or 10 µM), or the membrane permeable cAMP analog, dbcAMP (0, 0.1, 1.0, or 10 mM), and the steroid content in the media was examined. Results shown in Table 1 demonstrate that treatment of HCG, FK, and dbcAMP significantly increased testosterone and E_2 production by co-incubated theca and granulosa cells almost in a

Table 1 Effects of modulators of adenylate cyclase (forskolin) and protein kinase A (PKA; dibutyryl cAMP (dbcAMP)) on steroid production and inhibitors of adenylate cyclase (SQ22536), voltage-sensitive calcium channels (VSCCs; verapamil) and calmodulin (W-5) on human chorionic gonadotropin (HCG)-stimulated steroid production. Theca and granulosa cells of *Cyprinus carpio* were incubated in SF-DMEM containing various concentrations of modulators and/or inhibitors for 16 h at $23 \pm 1^\circ\text{C}$. Cells were pre-incubated for 1 h in the presence of inhibitors. Each value represents $\pm$ S.E.M. of five experiments, comprising three replicate incubations of follicle cells obtained from single donor fish

Stimulators/inhibitors	Doses	Testosterone (pg/ml)	17 β -estradiol (pg/ml)
Control	—	127 $\pm$ 13.6	130 $\pm$ 15.4
HCG (ng/ml)	25	348 $\pm$ 17.2*	470 $\pm$ 32.5*
	50	480 $\pm$ 24.3*	570 $\pm$ 37.9*
	100	740 $\pm$ 33.01 ^{*,a}	880 $\pm$ 78.3 ^{*,a}
	200	762 $\pm$ 35.02*	885 $\pm$ 69.9*
Forskolin (μM)	0.1	462 $\pm$ 35.4*	580 $\pm$ 46.1*
	1.0	670 $\pm$ 43.2*	770 $\pm$ 65.9*
	10	740 $\pm$ 53.2*	840 $\pm$ 63.5*
dbcAMP (mM)	0.1	426 $\pm$ 44.9*	520 $\pm$ 48.02*
	1.0	615 $\pm$ 76.8*	735 $\pm$ 72.49*
	10	660 $\pm$ 75.9*	800 $\pm$ 76.44*
HCG (100 ng/ml) + SQ22536 (mM)	0.1	655 $\pm$ 60.9	725 $\pm$ 69.4
	0.5	345 $\pm$ 31.0 ^b	440 $\pm$ 43.3 ^b
	1.0	222 $\pm$ 26.9 ^b	240 $\pm$ 31.8 ^b
HCG (100 ng/ml) + verapamil (μM)	0.1	540 $\pm$ 48.6 ^b	607 $\pm$ 57.5 ^b
	1.0	240 $\pm$ 25.1 ^b	250 $\pm$ 31.4 ^b
	2.0	180 $\pm$ 18.8 ^b	210 $\pm$ 29.5 ^b
HCG (100 ng/ml) + W-5 (μM)	0.1	630 $\pm$ 54.9	700 $\pm$ 69.5
	0.2	505 $\pm$ 43.3 ^b	580 $\pm$ 46.3 ^b
	1.0	370 $\pm$ 35.6 ^b	370 $\pm$ 54.3 ^b
	2.0	240 $\pm$ 30.8 ^b	240 $\pm$ 29.9 ^b
	10	215 $\pm$ 31.6 ^b	210 $\pm$ 29.5 ^b

* $P < 0.05$ versus without HCG, FK or dbcAMP; ^{a,b} means with different letters differ significantly from each other ($P < 0.05$).

concentration-dependent manner compared with their respective control values ($P < 0.05$). The optimal effective doses of HCG, FK, and dbcAMP for both the steroid productions were 100 ng/ml, 1 μM , and 1 mM respectively. The minimal tried concentrations at which they were able to induce steroid productions were 25 ng/ml, 0.1 μM , and 0.1 mM respectively.

Effects of inhibitors of adenylate cyclase, VSCCs, and calmodulin on HCG-stimulated steroid production

Follicle cells, after 6 h co-incubation in BSA-DMEM, were treated for 16 h in SF-DMEM with increasing concentrations of inhibitors of adenylate cyclase, SQ22536 (0, 0.1, 0.5, or 1.0 mM); VSCCs, verapamil (0, 0.1, 1.0, or 2.0 μM); or calmodulin, W-5 (0, 0.1, 0.2, 1.0, 2.0, or 10.0 μM), and HCG-stimulated steroid production was examined. It appears from Table 1 that SQ22536, verapamil, and W-5 at their increasing concentrations attenuated HCG-stimulated testosterone and E_2 production gradually and significantly ($P < 0.05$) after 16 h of incubation. The concentrations of SQ22536, verapamil, and W-5 at which maximum inhibition recorded were 1 mM, 2 μM , and 10 μM respectively (Table 1).

Effects of inhibitors of VSCCs and calmodulin on FK- and dbcAMP-stimulated steroid productions

Follicle cells, after 6 h co-incubation in BSA-DMEM, were treated for 16 h in SF-DMEM with either no treatment (control) or 1.0 μM FK or 1.0 mM dbcAMP to stimulate testosterone and E_2 production. Each agonist was tested with and without one of the two inhibitors: 2.0 μM verapamil or 10 μM W-5. Testosterone and E_2 production by co-incubated cells induced by the treatment with FK or dbcAMP was significantly attenuated when co-treated with 2.0 μM verapamil ($P < 0.05$; Fig. 1A). The calmodulin inhibitor W-5 at a concentration of 10 μM also significantly inhibited FK- and dbcAMP-induced testosterone and E_2 production ($P < 0.05$; Fig. 1B).

Effects of calcium ionophore A23187 on HCG-stimulated steroid production

Follicle cells, after 6 h co-incubation in BSA-DMEM, were treated for 16 h in SF-DMEM with either increasing concentrations of calcium ionophore A23187 alone (control) or HCG (50 ng/ml) plus increasing concentrations of A23187, and steroid production was estimated. From Fig. 2A and B, it appears that A23187 alone over a dose range of 0, 0.1,

Figure 1 Effects of inhibitors of VSCCs and calmodulin on forskolin- and dbcAMP-stimulated steroid productions by co-incubated theca and granulosa cells (A and B). Mean testosterone and 17β-estradiol production of cells incubated with either no treatment (control, C), 1.0 μM forskolin (FK), or 1.0 mM dbcAMP, each agonist with and without 2 μM verapamil (Ver) or 10 μM W-5 is shown. Each value represents $\pm$ S.E.M. of five experiments, comprises three replicate incubations of theca-granulosa cells obtained from single donor fish. Asterisk denotes values significantly ($P < 0.05$) different from those shown for without modulators (control). ^{a,b}Means with different letters differ significantly from each other ($P < 0.05$).

0.2, 0.6, 1.0, or 2.0 μM had a stimulatory effect on basal testosterone and E₂ production and showed significant stimulation at higher concentrations ($P < 0.05$). HCG-stimulated testosterone and E₂ production was gradually and significantly increased ($P < 0.05$) by A23187 at increasing concentrations with a maximal at 1.0 μM (Fig. 2A and B).

Effects of inhibitor of MEK1/2 on HCG-stimulated steroid production

A role for MAPK-activated signaling in HCG-stimulated testosterone and E₂ production by co-incubating theca and granulosa cells with HCG and MEK1/2 PD98059 was shown. For this, following 6 h co-incubation in BSA-DMEM, theca and granulosa cells were preincubated for 1.0 h with increasing doses of PD98059 (0.1, 1.0, and 5.0 μM) followed by incubation with HCG (100 ng/ml) for further 16 h, and steroid contents in the media were estimated. Figure 3A and B shows that

PD98059 at increasing concentrations gradually and significantly ($P < 0.05$) inhibited testosterone and E₂ production (75%) induced by HCG. At high concentration of PD98059 (5 μM), HCG-induced testosterone and E₂ production was not significantly different from their basal levels.

Effect of HCG on ERK1/2 phosphorylation and effect of inhibitor of MEK1/2

After 6 h incubation in BSA-DMEM and 2 h in SF-DMEM, media were replaced with fresh SF-DMEM and the cells were treated with increasing concentrations of HCG (0, 10, 25, 50, or 100 ng/ml) for 120 min or for 0, 15, 30, 60, and 120 min with HCG (100 ng/ml) or PD98059 (0, 0.1, or 1.0 μM). Immunoblot analysis of the cell lysate demonstrates that treatment of cells with increasing concentrations of HCG for 120 min induced a dose-dependent increase in the levels of phosphorylated ERK1/2 (Fig. 4A). Follicle cells treated with HCG for different times showed increasing levels of phosphorylated ERK1/2, the strongest response to HCG occurred between 60 and 120 min (Fig. 4B). The stimulatory effect of HCG on phosphorylated ERK1/2 was not attributed to increased ERK protein levels, as total ERK1/2 protein

Figure 2 Effects of calcium ionophore A23187 on HCG-stimulated steroid production by co-incubated theca and granulosa cells of common carp (A and B). Cells were incubated in 500 μl of serum-free DMEM for 16 h at 23 $\pm$ 1 °C with ionophore A23187 alone at different concentrations or with HCG (50 ng/ml). Each value represents $\pm$ S.E.M. of five experiments, comprises three replicate incubations of theca-granulosa cells obtained from single donor fish. Asterisk denotes values significantly ($P < 0.05$) different from those shown for without hormone or A23187 (0). ^{a,b}Means with different letters differ significantly from each other ($P < 0.05$).

Figure 3 Effects of inhibitors of MEK1/2 on HCG-stimulated steroid production by co-incubated theca and granulosa cells (A and B). Cells were incubated in 500 μl of serum-free DMEM for 16 h at $23 \pm 1^\circ\text{C}$ containing HCG (100 ng/ml) and increasing concentrations of PD98059. Each value represents $\pm$ S.E.M. of five experiments and comprises three replicate incubations of theca–granulosa cells obtained from single donor fish. ^{a,b}Means with different letters differ significantly from each other ($P < 0.05$).

was unaffected by incubation time and HCG treatment (Fig. 4A–C, lower panel). Follicle cells treated with HCG (100 ng/ml) for 60 and 120 min in the presence of increasing concentrations of PD98059 (0, 0.1, or 1.0 μM) blocked ERK1/2 phosphorylation almost in a concentration-dependent manner (Fig. 4C).

Effect of MEK inhibitor on FK- and dbcAMP-stimulated steroid productions

Follicle cells, after 6 h incubation in BSA–DMEM and 2 h incubation in SF–DMEM, were preincubated for 1 h with 1.0 μM of PD98059 followed by incubation with HCG (100 ng/ml), FK (1.0 μM), or dbcAMP (1.0 mM) for 16 h, and steroid contents in the media were estimated. It appears from Fig. 5A and B that PD98059 significantly ($P < 0.05$) attenuated the steroidogenic response to HCG, FK, and dbcAMP by 68, 62, and 60% respectively.

Effect of modulators of adenylate cyclase and PKA on ERK1/2 phosphorylation and effect of MEK inhibitor

Follicle cells, after 6 h incubation in BSA–DMEM and 2 h incubation in SF–DMEM, were treated for 1 h with PD98059 (1.0 μM) followed by incubation with HCG (100 ng/ml) or FK (1.0 μM) or dbcAMP

(1.0 mM) for 120 min, and ERK1/2 phosphorylation was examined. It appears from Fig. 6 that treatment with HCG or dbcAMP or FK for 120 min increased the levels of ERK1/2 phosphorylation in co-incubated theca and granulosa cell lysate of common carp. The MEK inhibitor PD98059 at concentration of 1.0 μM reduced basal levels of active ERK1/2 and blocked HCG-, dbcAMP-, and FK-induced increases in ERK1/2 phosphorylation (Fig. 6).

Effect of inhibitors of VSCCs and calmodulin on HCG-induced ERK1/2 phosphorylation

In a separate experiment we tested the ability of VSCCs blocker, verapamil, and calmodulin inhibitor, W-5, to modulate the HCG-stimulated and basal ERK1/2 activity in co-incubated theca and granulosa cells of common carp. For this, following 6 h incubation in BSA–DMEM and 2 h in SF–DMEM, cells were treated for 1 h with 1.0 μM PD98059 followed by incubation with 2 μM verapamil, 10 μM W-5 with or without HCG (100 ng/ml) for 120 min, and ERK1/2 phosphorylation was determined. It appears from Fig. 7 that ERK1/2 phosphorylation induced by HCG was sufficiently blocked by the treatment with verapamil (2.0 μM) and W-5 (10 μM).

Figure 4 Concentration-, time-, and MEK1/2-dependent activations of ERK1/2 by HCG in co-incubated theca and granulosa cells. Immunoblot analyses of phosphorylated ERK1/2 (P-ERK1/2) and total ERK1/2 (T-ERK1/2) in cells incubated with increasing concentrations of HCG for 120 min (A), with 100 ng/ml HCG for 15–120 min (B), or with 100 ng/ml HCG and increasing concentrations of PD98059 for 60 min (C). Mobilities of molecular mass standard are given in kDa on the left (A). Immunoblot analyses were performed at least three times with nearly identical results.

Figure 5 Effects of inhibitor of MEK1/2 on forskolin (FK)- and dbcAMP-stimulated steroid productions by co-incubated theca and granulosa cells (A and B). Cells were incubated in 500 μ l of serum-free DMEM for 16 h at 23 ± 1 °C containing HCG (100 ng/ml) and PD98059 (1.0 μ M). Each value represents $\pm$ s.e.m. of five experiments and comprises three replicate incubations of theca-granulosa cells obtained from single donor fish. ^{a,b}Means with different letters differ significantly from each other ($P < 0.05$).

Discussion

In this paper, we demonstrate that calcium-mediated cell signaling is important in regulating gonadotropin-induced testosterone and E_2 production by short-term co-incubated theca and granulosa cells of *C. carpio*. Inhibition of steroid production in the presence of an L-type calcium channel blocker demonstrates that calcium influx from extracellular store is required for gonadotropin-stimulated steroidogenesis in ovarian follicles. Furthermore, inhibition of HCG-stimulated steroid production in the presence of a calmodulin inhibitor indicates that this calcium-binding protein is also involved in such processes. These results corroborate earlier findings with either whole ovarian follicles or isolated follicle cells of fish (Mukherjee *et al.* 2001, Benninghoff & Thomas 2005, 2006a) and other vertebrates (Van Der Kraak & Wade 1994). We further observed that addition of calcium ionophore A23187, which elevates intracellular calcium levels, was sufficient

to increase steroid production by co-incubated follicle cells in the absence of gonadotropin. A similar stimulatory role of calcium ionophores on basal steroid production has also been reported in fish and other vertebrates (Srivastava & Van Der Kraak 1994, Benninghoff & Thomas 2005). The stimulatory effects of A23187 on HCG-induced testosterone and E_2 production by cells of postvitellogenic ovarian follicles in our study further indicate a regulatory role of intracellular calcium in HCG-induced ovarian steroidogenesis in this species. Taken together, all these data indicate the involvement of calcium-dependent signaling in ovarian steroidogenesis in common carp.

Consistent with observations in other vertebrates and in fish (reviewed in Leung & Steele (1992) and Benninghoff & Thomas (2006a)), increased steroid production by co-incubated carp ovarian follicle cells in presence of FK and dbcAMP, the modulators of adenylate cyclase and PKA respectively, and inhibition of HCG-stimulated steroid production in the presence of a specific adenylate cyclase inhibitor, SQ22536, demonstrate the regulatory role of adenylate cyclase and PKA in gonadotropin-induced ovarian steroidogenesis in such fish. Although conflicting reports are available on the requirement of calcium ion in gonadotropin-stimulated cAMP production by rat and bovine granulosa cells (Tsang & Carnegie 1984, Davis *et al.* 1987), reports with other mammals indicate that action of HCG to increase cAMP production requires the presence of calcium (Veldhuis & Klase 1982, Asem & Hertelendy 1986). The possible target for calcium is the adenylate cyclase, and increased intracellular calcium concentrations have both positive and negative effects on adenylate cyclase leading to increased and decreased production of cAMP (Jamaluddin *et al.* 1992, Srivastava & Van Der Kraak 1994). Requirement of calcium ion in HCG-induced cAMP production by Atlantic croaker ovarian follicles has also been reported (Benninghoff & Thomas 2006a).

Cross talk among various signal transduction systems, including adenylate cyclase- and calcium-dependent signaling pathways, has been demonstrated in many cell types and in response to a variety of receptor

Figure 6 Effects of modulators of adenylate cyclase and PKA on ERK1/2 activation in co-incubated theca and granulosa cells. Immunoblot analyses of phosphorylated ERK1/2 (P-ERK1/2) and total ERK1/2 (T-ERK1/2) in cells incubated with 100 ng/ml HCG or 1.0 μ M forskolin (FK) or 1.0 mM dbcAMP with or without 1.0 μ M PD98059 for 120 min are shown. Immunoblot analyses were performed at least three times with nearly identical results.

Figure 7 Effects of inhibitors of VSCCs and calmodulin on HCG-stimulated ERK1/2 phosphorylation in co-incubated theca and granulosa cells. Immunoblot analyses of phosphorylated ERK1/2 (P-ERK1/2) and total ERK1/2 (T-ERK1/2) in cells incubated with HCG (100 ng/ml) with or without either verapamil (2 μM) or W-5 (10 μM) for 120 min are shown. Immunoblot analyses were performed at least three times with nearly identical results.

agonists (Rasmussen 1981, Bygrave & Roberts 1995, Richards 2001). In this study, we could not measure cAMP levels after HCG treatment, but increased basal and HCG-, as well as FK-, and dbcAMP-induced steroid productions in the presence of both extracellular and intracellular calcium may also suggest similar cross talk between adenylate cyclase/PKA and calcium in HCG-induced ovarian steroidogenesis in common carp.

Earlier studies both in fish and other vertebrates demonstrate a regulatory role of calcium and calmodulin distal to activation of adenylate cyclase and PKA (Kleis-San Francisco & Schuetz 1988, Van Der Kraak 1991, Benninghoff & Thomas 2006a). In this study, we also provide evidence for a regulatory role of calcium or calmodulin distal to activation of adenylate cyclase and PKA using inhibitors of VSCCs or calmodulin to block FK- and dbcAMP-stimulated steroid productions in the carp ovarian follicle cells. Requirement of active CaMK for full steroidogenic response to FK and dbcAMP in croaker ovarian follicle has also been demonstrated (Benninghoff & Thomas 2006a). Thus, like other vertebrates, in common carp ovarian steroidogenesis, calcium and cAMP appear to act as dual second messenger molecules activating separate signaling pathways that may converge at a site distal to PKA activation.

The results of this study clearly show a third signaling pathway involving MEK1/2 and ERK1/2 in gonadotropin-induced steroidogenesis in common carp ovary. We observed that HCG treatment increased ERK1/2 phosphorylation in theca-granulosa cell lysate of common carp ovary almost in a dose- and time-dependent manner. Involvement of the MAPK pathway in gonadotropin-induced ovarian steroidogenesis in fish has recently been demonstrated for the first time in Atlantic croaker by Benninghoff & Thomas (2006b), and to our knowledge, demonstration of involvement of MAP kinase signaling in gonadotropin-stimulated steroidogenesis in common carp ovarian follicles may be the second one in any nonmammalian vertebrates. A role for MAPK/ERK signaling in regulating gonadotropin-induced steroidogenesis in mammalian and hen

granulosa cells (Moore *et al.* 2001, Seger *et al.* 2001, Dewi *et al.* 2002, Cottom *et al.* 2003, Su *et al.* 2006, Woods & Johnson 2007) and also in rat Leydig cell (Martinelle *et al.* 2004) has been reported. Thus, fish being evolutionarily distant from mammal share a common signaling pathway in mediating gonadotropin-induced ovarian steroidogenesis.

We, in this study, further observed that HCG-stimulated ERK1/2 phosphorylation leading to increased production of testosterone and E₂ is significantly inhibited by an MEK1/2 inhibitor PD98059, suggesting that the action of HCG on ERK1/2 phosphorylation is mediated by the upstream MEK1/2. Similar effects of PD98059 and another MEK1/2 inhibitor, U-0126, on HCG-induced follicular cell steroidogenesis were observed in Atlantic croaker (Benninghoff & Thomas 2006b). Although involvement of MAPK in mediating gonadotropin-stimulated steroidogenesis has been observed in many species, conflicting results in different steroidogenic tissues have been demonstrated (Seger *et al.* 2001, Dewi *et al.* 2002, Manna *et al.* 2002, 2006, Seto-Young *et al.* 2003, Martinelle *et al.* 2004, Tajima *et al.* 2005). For example, inhibition of MAPK/ERK1/2 activity with PD98059 and U0126 has been shown to be associated with stimulation (Seger *et al.* 2001, Tajima *et al.* 2003), inhibition (Gyles *et al.* 2001, Manna *et al.* 2002, Martinelle *et al.* 2004), or no effect (Tai *et al.* 2001, Seto-Young *et al.* 2003, Tajima *et al.* 2005) on steroidogenic response. Taken together, these findings demonstrate a complex role for the MAPK/ERK cascade in the regulation of the steroidogenic response that appeared to be tissue- and stimulus specific. The mechanism by which gonadotropin binding to its GPCRs triggers activation of the MEK/ERK pathway is still controversial. As suggested by previous workers (Pierce *et al.* 2001, Kim *et al.* 2002, Drube *et al.* 2006, Evaul & Hammes 2008), it may be possible that gonadotropin binding to GPCRs in carp ovarian follicle cells activates the MEK/ERK pathway through trans-activation of EGFRs and further studies would require exploration of the actual mechanism of such trans-activation.

Involvement of cAMP in HCG-stimulated activation of ERK in carp ovarian follicle is demonstrated by showing increased ERK1/2 phosphorylation in presence of FK and dbcAMP. Similar cAMP-dependent ERK1/2 phosphorylation has been demonstrated in mammalian granulosa cells (Seger *et al.* 2001, Dewi *et al.* 2002, Cottom *et al.* 2003) and also in croaker ovarian follicles (Benninghoff & Thomas 2006a). Attenuation of FK- and dbcAMP-stimulated ERK1/2 phosphorylations and steroid production by the MEK1/2 inhibitor PD98059 indicate that the function of the MEK/ERK pathway is likely to be distal to adenylate cyclase and PKA. As we have not used any PKA inhibitor in our study, involvement of PKA in HCG-stimulated ERK1/2

Figure 8 Model for signal transduction pathways regulating steroidogenesis in carp theca and granulosa cells. Gonadotropins (LH or HCG) activate GtHR, leading to increase in cAMP levels and subsequent PKA activity. Increase in PKA signaling regulates steroidogenesis. Calcium-dependent signaling pathways are also involved in the regulation of steroidogenesis. Increase in cAMP may also lead to the activation of MEK/ERK pathway independent of PKA through activation of cAMP-dependent guanine nucleotide exchange factor (cAMP-GEF). cAMP-GEF may in turn trigger small GTPases, such as Rap-1 and Rap-2, that are known to activate the MAP kinase cascade. Interactions among signaling pathways were observed as demonstrated by positive effects of elevated intracellular Ca^{2+} level on adenylate cyclase and the reduction of forskolin- or dbcAMP-mediated steroid production by inhibitors of VSCCs and calmodulin. Calcium/calmodulin-dependent signaling pathways also appear to mediate gonadotropin-induced ERK activation. Solid line indicates pathways investigated in this study. Dotted lines indicate pathways extensively studied in other vertebrate models. Dashed lines depict possible targets of regulation by these signaling pathways. AC, adenylyl cyclase; CaM, calmodulin; CaMK, calcium/calmodulin-dependent kinases; dbcAMP, dibutyryl cAMP; ER, endoplasmic reticulum; ERK, extracellular signal-regulated protein kinase; GtH, gonadotropin; GtHR, gonadotropin receptor; IP3, inositol triphosphate; MEK, mitogen-activated protein kinase kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PKA, cAMP-dependent protein kinase; PLC, phospholipase C; StAR, steroidogenic acute regulatory protein; VSCC, voltage-sensitive calcium channel. Full color version of this figure available via <http://dx.doi.org/10.1677/JME-10-0061>.

phosphorylation in this species is not clear. Earlier studies with croaker ovarian follicles suggested that the stimulatory effect of HCG on ERK phosphorylation is not mediated through PKA (Benninghoff & Thomas 2006a). On the contrary, involvement of PKA in gonadotropin-induced ERK activation has been demonstrated in rat and human granulosa cells (Seger *et al.* 2001, Dewi *et al.* 2002). One mechanism that activates the ERK cascade independent of PKA includes activation of cAMP-responsive guanosine nucleotide exchange factor for small GTPase such as Rap-1 and Rap-2. On binding with cAMP, these components rapidly activate Rap-1, which then promotes the activation of B-Raf and the rest of the ERK cascade (de Rooij *et al.* 1998, Richards 2001).

Some recent studies demonstrated a role for calcium, calmodulin, or CaMKs in the regulation of MAPK activity in a variety of cells (Agell *et al.* 2002,

Gomez *et al.* 2002, Stocco *et al.* 2002). Cottom *et al.* (2003) described that FSH induction of ERK activity in rat granulosa cells is mediated in part by calcium influx from extracellular stores and increases in cytosolic calcium induce ERK phosphorylation. In this study, clear inhibition of HCG-stimulated ERK1/2 phosphorylation in the presence of an antagonist of VSCCs and calmodulin indicates that HCG-induced ERK activity in such cells is likely to be mediated by calcium-dependent signal transduction. In Atlantic croaker ovarian follicles, however, HCG-induced ERK activity is not mediated by calcium-calmodulin-dependent signal transduction (Benninghoff & Thomas 2006b). Thus, a novel role of calcium and calmodulin in the activation of the MAP kinase signaling cascade involving MEK1/2 and ERK1/2 in the regulation of gonadotropin-induced steroidogenesis has been identified for the first time in fish ovarian follicles. It is therefore

possible that the major signaling pathway regulating steroidogenesis identified in this study including adenylate cyclase-, calcium-, and MAPK-dependent pathways converges at a point distal to activation of PKA and ERK1/2. The possible target for coordinate regulation of this signaling pathway may be the steroidogenic acute regulatory protein and/or transcription factor regulating its synthesis because there is evidence that in steroidogenic cells, cAMP, PKA, calcium, CaMK, and MAPK can modulate the activity of this protein or associated transcription factors (Cherradi *et al.* 1997, Manna *et al.* 1999, Gyles *et al.* 2001, Tajima *et al.* 2003, Evaul & Hammes 2008).

The present result together with a previous finding demonstrating calcium-dependent regulation of testosterone production (Benninghoff & Thomas 2006b) shows that multiple but independent signaling pathways are operative in gonadotropin-induced ovarian steroidogenesis in carp ovarian follicles. This is summarized in the model of Fig. 8. The role of MAP kinase signaling cascade involving MEK1/2 and ERK1/2 in the regulation of gonadotropin-induced steroid production by co-incubated theca and granulosa cells has been identified. Furthermore, a novel role of calcium and calmodulin in the activation of the MAP kinase signaling cascade involving MEK1/2 and ERK1/2 in the regulation of gonadotropin-induced steroidogenesis has also been identified and there is evidence for a cross talk between the adenylate cyclase, calcium/calmodulin, and MAP kinase pathways in this process.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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