Stimulation of salmon calcitonin on secretion of 17 β -estradiol by the ovarian follicles of common carp, *Cyprinus carpio*

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Abstract

The effects of salmon calcitonin (sCT) on the secretion of 17 β -estradiol (E₂) were examined in female common carp, *Cyprinus carpio*. Vitellogenic stage fish adapted to high-Ca water were i.p. injected with vehicle, sCT, human chorionic gonadotropin (hCG), or hCG plus sCT. To determine whether ovarian follicles are equipped with CT receptors, a CT binding assay was conducted. In the *in vitro* experiments, vitellogenic follicles were incubated with stimulators and inhibitors. Administration of sCT increased the basal and hCG-stimulated E₂ release *in vivo* and *in vitro*. Binding characteristics of [¹²⁵I]sCT to plasma membrane preparation of carp ovarian follicles showed saturability with high-affinity ($K_d = 48.48$ pmol/l and $B_{max} = 1.2$ pmol/mg protein). To clarify the mechanism of E₂ production by sCT, *in vitro* effect of sCT and hCG on aromatase activity (conversion of testosterone to E₂) and cytochrome P450 aromatase

(P450arom) gene expression in carp ovarian follicles were investigated. Salmon CT-stimulated both aromatase activity and P450arom gene expression in ovarian follicles of carp. sCT-stimulated E₂ release by the ovarian follicles *in vitro* was augmented in the presence of dibutyryl cAMP. Inhibitor of protein kinase A (PKA), SQ 22536 inhibited sCT-stimulated steroid production in a dose-dependent manner. Specific inhibitor of protein kinase C (PKC), NPC-15437 dihydrochloride had no inhibitory effects on sCT-induced E₂ release. The present study indicates that sCT binds specifically to carp ovary and stimulates E₂ production by increasing the activity of cytochrome P450 aromatase and P450arom gene expression. The results further suggest that stimulatory action of sCT on E₂ production is mediated through cAMP pathway. *Journal of Endocrinology* (2008) **196**, 413–424

Introduction

Calcitonin (CT), a 32-amino acid peptide, is synthesized by the C cells of mammalian thyroid gland (Copp *et al.* 1962, Foster *et al.* 1964) and ultimobranchial gland of non-mammalian vertebrates (Copp *et al.* 1967). The classic concept of CT function in mammals has focused on its effects on calcium homeostasis (Azria 1989). By comparison, the role of CT on calcium homeostasis in fish has long been controversial. Conflicting results concerning hypocalcemic effect of CT have been reported, depending on the species and protocol followed (Chan *et al.* 1968, Yamauchi *et al.* 1978, Wendelaar Bonga & Pang 1991). This situation changed completely when gills were recognized as one of the many target organs for CT action in fish (Milhaud *et al.* 1977). Some recent information indicated that CT exerts its hypocalcemic action in fish through inhibition of gill calcium transport (Wagner *et al.* 1997, Mukherjee *et al.* 2004a). We also demonstrated that as in mammals, hypocalcemic effect of CT in certain teleost is exerted through bone calcium resorption (Mukherjee *et al.* 2004b).

In addition to the involvement of CT in calcium homeostasis, its action on brain, pituitary, and gonad have been investigated in mammals. Specific CT-binding sites have been found in the brain and pituitary gland (Fischer *et al.* 1981, Maurer *et al.* 1983) and in the ovary and testis (Chausmer *et al.* 1982, George *et al.* 1997). In addition to the thyroid, CT-like immunoreactivity has been found in the pituitary gland of humans and rats (Cooper *et al.* 1980). Wang *et al.* (1994) found that CT-like peptide including human CT, sCT, and CT gene-related peptide, inhibit the spontaneous and gonadotropin-stimulated testosterone secretion by acting directly at the testes and reducing the release of pituitary luteinizing hormone (LH). Inhibition of sCT on secretion of progesterone and gonadotrophin-releasing hormone (GnRH)-stimulated release of pituitary LH hormone in rats has also been documented (Tsai *et al.* 1999). Reports are available that plasma CT exhibited a peak concentration on the day of diestrus and reduced to the lowest on the day of estrus (Cressent *et al.* 1983). All these observations indicate an endocrine role of endogenous CT at the brain, pituitary, and gonad in mammals.

From the beginning of the study on the effects of CT in fish, its action on the regulation of reproduction has been proposed by many workers. Evidence is available on high plasma CT levels during peak spawning season in Coho salmon, Japanese eel, and rainbow trout (Defetos *et al.* 1974, Yamauchi *et al.* 1978, Bjorsson *et al.* 1986). Increase in plasma CT level by the induction of E₂ has been reported in Coho salmon (Bjorsson *et al.* 1989). Available information suggests that CT is involved in mobilizing calcium or in directing its mobilization by protection of a certain calcium pool during vitellogenesis (Bjorsson *et al.* 1989). Suzuki *et al.* (2004) described that E₂ acts on UBG to induce the release of CT, which in turn may play an important role in reproduction directly and or indirectly through calcium. Although these studies indicate a relationship between CT and fish reproduction they fail to explain the exact link between them. However, no effort has yet been made in fish to study the effect of CT, if any, at the ovarian level directly or at the brain-pituitary levels to modulate the reproduction indirectly.

The present study makes an attempt to examine the effects of sCT on the basal and hCG-stimulated *in vivo* release and *in vitro* production of E₂ by ovarian follicles of *Cyprinus carpio*. To determine whether fish ovarian follicles are equipped with functional CT receptors, a CT-binding assay was conducted. Cytochrome P450arom is the key enzyme for conversion of testosterone to E₂ in the granulosa cells. The P450arom mRNA levels are increased in association with increases of enzyme activity during vitellogenesis in teleost (Fukada *et al.* 1996, Kagawa *et al.* 2003). Therefore, the aim of the present study was to elucidate the role of sCT on aromatase activity and P450arom gene expression in the ovarian follicles of common carp. Furthermore, we have carried out experiments which show that signal transduction of the stimulatory effects of sCT on E₂ release by the ovarian follicles may be operated through cAMP pathway.

Materials and Methods

Animals

Adult female common carp *C. carpio* (300–400 g body wt), collected from a local fish farm during the months of September and October were maintained in recirculating dechlorinated normal tap water in laboratory concrete tanks (300 l capacity, Ca²⁺, 0.15 mM) at 23 $\pm$ 2 °C for 5 days. They were fed with commercial fish food (Shalimar Fish Food; Bird and Fish food manufacturer, Mumbai, India). Fish were then transferred to high-calcium water (number of fish = 120, Ca²⁺, 0.4 mM) for 7 days before treatment.

During the months of September and October in the plains of West Bengal, India, the ovary of female common carp comprises mostly of vitellogenic follicles (0.3–0.4 mm diameter) with oocytes containing centrally located germinal vesicle. The cytoplasm was filled with yolk granules and cortical granules were shown to cover the entire oocyte. Follicular developmental

stage was determined by stripping out a few follicles through the ovipore followed by examination under microscope after fixing them with a clearing solution of acetic acid–ethanol–formalin mixture (1:6:3 v/v) for 12 h.

Chemicals

Synthetic sCT, (Lot no. 118H49611), all cold steroids, and dibutyryl cyclicAMP (dbcAMP) were purchased from Sigma Chemicals. Human chorionic gonadotropin (hCG) was a gift from National Hormone and Pituitary Program (Torrance, CA, USA). SQ 22536 (RBI, Natick, MA, USA) and NPC-15437 dihydrochloride (Sigma) were gifts from Dr Arun Bandopadhyay, Molecular Endocrinology Laboratory, Indian Institute of Chemical Biology, Kolkata. Triclanemethane sulfonate (MS 222) was a gift from Sandoz Basels, Switzerland. Total RNA isolation (TRI) reagent was purchased from Ambion Inc., Foster City, CA, USA. Smart-PCR cDNA synthesis kit was purchased from Clontech. RevertAid M-MuLV reverse transcriptase and deoxyNTPs were procured from MBI Fermentas and Taq DNA polymerase from Invitrogen. Labeled steroids, [³H]estradiol-17 β (sp. activity 75.0 Ci/mmol), [³H]testosterone (sp. activity 95.0 Ci/mmol), and [¹²⁵I]sCT (Code IM250, sp. activity 2000 Ci/mmol) were procured from Amersham Biosciences. The E₂ antibody was a gift from Prof. Gordon Niswender, Colorado State University, Fort Collins, CO, USA. All other chemicals used were of analytical and molecular biology grade.

Effects of sCT on plasma E₂ levels

Vitellogenic stage fish, after being maintained for 7 days in high-Ca²⁺ water, were given i.p. a single injection of increasing concentration of sCT in such a way that each fish received 0.1, 0.5, 1.0, or 2.0 μ g sCT/100 g body wt. Controls were injected with vehicle (0.6% aqueous saline and 1% gelatin preparation). Sampling of fish was done 8 h after injection. In another experiment, a group of fish was given single injections of sCT (0.5 μ g/100 g body wt) or hCG (0.5 μ g/100 g body wt) or hCG plus sCT (each 0.5 μ g/100 g body wt) at 0700 h in the morning. Controls were injected with vehicle. Fish were sampled at 0, 2, 4, 8, 12, 16, and 24 h after injection. The volume of vehicle in both the experiments was 20 μ l per fish. Fish were lightly anesthetized with MS 222 (1:1000, pH 7.4) before injection. Immediately after sampling, blood was collected from the caudal vein under light anesthesia, processed for plasma separation, and kept at –20 °C until steroid analysis.

In vitro incubation of ovarian follicles

The donor fish (vitellogenic stage) selected for ovarian follicles were killed by decapitation at 0700 h in the morning and ovaries were placed in ice-cold Idler's medium containing streptomycin (100 μ g/ml) and penicillin (100 IU/ml) adjusted to pH 7.4 (Mukherjee *et al.* 2006). Follicles, after

collection were kept separately in ice-cold medium until use. ~100 mg follicles were initially placed in individual wells of a 24-well culture plate (Tarson, Kolkata, India) for 2 h that contained 1.0 ml control medium. This 2-h pre-incubation time was required to waive the surgical shock (Mukherjee *et al.* 2006). After 2 h, the medium was replaced with fresh medium containing stimulators and inhibitors. Inhibitors were added 1 h prior to the addition of test compounds. Cultures were placed in a metabolic shaker bath at $23 \pm 1^\circ\text{C}$ under air. Viability of ovarian follicles was observed to be about 90% as detected using 0.1% Trypan blue dye exclusion. At the end of incubation, medium samples were aspirated, centrifuged (5000 g), and stored at -20°C for E_2 measurement by specific RIA. Aromatase activity in the ovarian follicles in response to sCT and hCG was estimated by *in vitro* conversion of labeled testosterone to labeled E_2 using the method of Chan & Tan (1986). For this, ovarian follicles (100 mg) were incubated in the absence or presence of sCT (50 ng/ml) or hCG (50 ng/ml) for 12 h at $23 \pm 1^\circ\text{C}$. All incubations contained 140 pmol ^3H testosterone (1×10^6 c.p.m., sp. activity 5400 mCi/mmol).

RNA isolation and cDNA preparation

Total RNA was extracted from isolated ovarian follicles (in both control and treated groups) using TRI Reagent solution following the manufacturer's instruction and the method described earlier (Chomczynski & Sacchi 1987) and cDNA was synthesized using Smart-PCR cDNA synthesis kit following the manufacturer's instruction.

RT-PCR

First-strand cDNA synthesis was carried out with 2 µg total RNA using RevertAid M-MuLV reverse transcriptase. To the tube oligo(dT)₁₈ primer, reverse transcription reaction buffer, RNAase inhibitor, deoxyNTPs were mixed (final volume 20 µl) and incubated at 42°C for 1 h for first-strand cDNA synthesis. From the cDNA prepared, 2 µl were used as template for RT-PCR with gene-specific primer, and relative expression was observed with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer (Roy *et al.* 2003). A 50 µl PCR volume was made by adding 2.5 U *Taq* DNA polymerase to a PCR mixture containing $1 \times$ reaction

buffer (50 mM KCl, 10 mM Tris-HCl (pH 8.3), 0.1% Triton-X-100, and 2.5 mM MgCl_2), 200 µM of each deoxyNTPs and 20 pmol of each primer. The PCR was performed for 35 cycles of denaturation at 94°C for 30 s (5 min in the first cycle), annealing at specific temperature for each set of primers for 30 s, and extension at 72°C for 30 s (10 min in the last cycle; Applied Biosystems, Foster City, CA, USA). The RT-PCR products were cloned, sequenced, and used for the expression purpose. The primers (used for RT-PCR) of the respective genes with the accession number and their amplified segments are listed in Table 1.

Membrane preparation of ovarian follicles for sCT binding assay

Membrane was prepared from ovarian follicle using the method of Birnbaumer & Swartz (1982) with few modifications. To state briefly, ovarian follicles after isolation were washed three to four times with chilled Idler's medium, weighed and homogenized in sodium phosphate buffer (0.01 mol/l, pH 7.4) under ice for 30 s. The homogenate was passed through single layer cheese-cloth to remove fat and cell debris and then spun at 3000 g in a refrigerated centrifuge for 10 min. The 3000 g pellet was re-centrifuged at 20 000 g for 30 min at 4°C . After washing, the pellet was re-suspended in phosphate buffer (0.01 mol/l, pH 7.4) in the ratio of 1 g/10 ml and stored at -70°C until use. Protein content of the preparation was measured according to the methods of Lowry *et al.* (1951) using BSA as the standard.

sCT binding assay

The incubation medium used for binding assays contained 5 mM MgCl_2 /l, 0.1 mol sucrose/l, and 0.1% (w/v) BSA in phosphate buffer (0.01 mol/l, pH 7.4). For [^{125}I]sCT binding, membrane preparation (2.0 mg protein) was incubated with 20 µl [^{125}I]sCT solution ($\sim 1 \times 10^5$ c.p.m.) in the absence (total binding) or presence of a 1000-fold excess of unlabeled sCT to measure nonspecific binding. Final assay volume was 500 µl at 23°C . Incubation was terminated at 90 min by addition of bovine γ-globulin (0.1% v/v) and NaCl (0.1 mol/l). Ice-cold polyethylene glycol (PEG; 1 ml, 20% w/v) was then added

Table 1 Primers used in semi-quantitative RT-PCR

	Forward primer	Reverse primer	Size of amplicon (bp)
Gene product			
CYP19A (cytochrome P450 aromatase; DQ534411)	5' TACACATTCTGGAGAGTTTATCA 3'	5' GGAAGTTGTCTAGACTGAACAT 3'	198
GAPDH (AJ870982)	5' AGGGGCTCAGTATGTTGTGG 3'	5' AGGAGGCATTGCTGACAACT 3'	185

to each tube under ice. The contents of these tubes after vortex were centrifuged at 1500 g for 10 min in a refrigerated centrifuge followed by aspiration of the supernatant. Pellets were then rinsed twice with 1.0 ml chilled assay buffer and centrifuged. The final pellets were counted in a [125 I] γ counter. Specific binding was estimated by subtracting nonspecific binding from total binding.

Extraction and assay of steroids

The method of extraction of E₂ from plasma and incubation medium was similar to the previously described procedure for this steroid (Mukherjee *et al.* 2001, Sen *et al.* 2002). Anti-E₂ serum was highly specific and cross-reacted with E₂, testosterone, 17 α -hydroxyprogesterone, and cortisol at 100, 1, <0.01, <0.01, and <0.01 respectively. The sensitivity of the assay was 12 pg/ml. Intra-assay and inter-assay coefficient of variation were 9 and 12% respectively.

Statistical analysis

All data were expressed as means $\pm$ S.E.M. Data from each experiment were subjected to one-way ANOVA followed by Bonferroni's multiple comparison tests. The level of significance chosen was $P < 0.05$.

Results

Plasma calcium and 17 β -estradiol (E₂) levels in response to sCT and hCG

Results shown in Fig. 1A demonstrate that sCT in increasing doses (0.1, 0.2, 0.5, 1.0, or 2.0 μ g/100 g body wt) caused a gradual rise in plasma E₂ levels in carp 8 h after injection. The maximum effective dose was 0.5 μ g. The minimum dose at which sCT was able to induce an increase in plasma E₂ levels was 0.1 μ g.

Intraperitoneal injection of vehicle did not alter the levels of plasma E₂ in carp (Fig. 1B). After 2 h of sCT injection (0.5 μ g/100 g body wt), the mean concentration of plasma E₂ increased by 22% and a maximum (77.92%) was recorded at 8 h followed by a gradual decline leading to basal plasma value at 24 h. Injection of hCG- (0.5 μ g/100 g body wt) stimulated E₂ release and the highest plasma E₂ concentration was recorded at 12 h (Fig. 1B). Injection of hCG plus sCT (each 0.5 μ g/100 g body wt) resulted in a significantly higher level of plasma E₂ at 8 h and 12 h after challenge when compared with that induced by hCG alone ($P < 0.05$, Fig. 1B).

Mean levels of total and ultrafiltrable plasma calcium in vitellogenic stage fish at all time points were 3.8 ± 0.4 mM and 1.6 ± 0.17 mM, respectively, for the vehicle-injected group, 3.75 ± 0.34 mM and 1.6 ± 0.15 mM, respectively, for the hCG-injected group, and 3.85 ± 0.36 mM and 1.65 ± 0.17 mM, respectively, for the sCT-injected group.

Figure 1 Dose-response effects of (A) salmon calcitonin (sCT) and a time-course study on the effects of (B) sCT (0.5 μ g/100 g body wt), human chorionic gonadotropin (hCG, 0.5 μ g/100 g body wt), and hCG+sCT (each 0.5 μ g/100 g body wt) on plasma concentration of 17 β -estradiol (E₂) in vitellogenic stage *C. carpio*. (A) Fish were injected with increasing doses of sCT as indicated and killed 8 h after injection. (B) Fish were given a single i.p. injection of sCT, hCG or sCT+hCG. Blood samples were collected from caudal vein at time indicated after hormone challenge. Each value (A and B) is mean $\pm$ S.E.M. of five observations (SC, saline control, (A) * $P < 0.05$ versus sCT at 0 μ g/100 g body wt, (B) + $P < 0.05$ versus hCG at 0.5 μ g/100 g body wt).

No significant differences in plasma calcium levels were observed among these three groups.

sCT binding to fish ovarian tissue

Membrane preparation (2.0 mg protein) from fish ovarian follicles was subjected to incubation with [125 I]sCT where radiolabeled sCT was added in increasing concentrations (0.5–4.0 nM). Figure 2A shows that with increasing concentrations of [125 I]sCT, the specific binding increases until 2 nM and then saturation was reached. Scatchard plot analysis of the data (Fig. 2B) showed that $B_{\max}$ (MBC) of ovarian follicular membrane preparation for [125 I]sCT was 1.2 pM/mg protein and K_d was 48.8 pM/l.

Figure 2 (A) Saturation curve of [¹²⁵I]sCT binding to membrane preparation of ovarian follicles of vitellogenic stage *C. carpio*. Specific binding was determined by subtracting the nonspecific binding from the total binding. (B) Scatchard plot of [¹²⁵I]sCT binding to fish ovarian follicular membrane preparation. (A and B) Values are $\pm$ S.E.M. of four observations taking ovarian follicles in duplicate from four donor fish.

Effects of sCT on E₂ production by the ovarian follicles in vitro

Since there was a significant increase in plasma E₂ levels in vitellogenic stage fish after sCT injection, and as sCT binds with the follicular membrane preparation with high-affinity, physiological importance of sCT binding to ovarian follicles was assessed by incubating follicles with varied concentrations of sCT without or with hCG and steroid production was measured.

The effect of sCT ranging from 25 to 200 ng/ml incubation on E₂ release by the ovarian follicles is illustrated in Fig. 3A. During a 12-h incubation, sCT at 50 ng dose released maximum quantity of E₂ in the medium ($P < 0.05$). Higher doses over 50 ng/incubation had no additive effects (Fig. 3A). Incubation of ovarian follicles with increasing doses of hCG (10–100 ng/ml incubation) for 12 h caused a significant increase of E₂ release at 25 ng dose and the highest was recorded at 50 ng/ml incubation

Figure 3 Dose-response effects of (A) sCT and (B) hCG on *in vitro* release of 17 β -estradiol by the vitellogenic follicles of *C. carpio*. (C) Dose-response effects of sCT on basal (hollow bar) and hCG-stimulated (solid bar) *in vitro* release of 17 β -estradiol by the vitellogenic follicles of *C. carpio*. All incubations were terminated at 12 h after addition of test compounds. Each value represents $\pm$ S.E.M. of five incubations taking follicles in triplicate from five donor fish ((A and B) * $P < 0.05$ versus tissues incubated without hormone (0), (C) * $P < 0.05$ versus sCT at 0 ng/ml and ⁺ $P < 0.05$ versus hCG at 0 ng/ml).

(Fig. 3B). Ovarian follicles were incubated with sCT (0–100 ng/ml) in the presence of hCG (25 ng/ml) for 12 h. Figure 3C shows that hCG-induced release of E₂ was significantly increased ($P < 0.05$) by sCT ranging from 25 to 100 ng dose/ml incubation. Ovarian follicles were incubated

with sCT or hCG (each 50 ng/ml) for various lengths of time up to 16 h. It appears from Fig. 4A that after addition of hormones E₂ release increased steadily from 2 h and the maximum was recorded at 6 h by sCT and 12 h by hCG.

Effects of sCT on P450 aromatase activity

Aromatase activity, which was estimated by *in vitro* conversion of labeled testosterone to labeled E₂ in the ovarian follicles, were significantly stimulated by sCT and hCG at a concentration of 50 ng/ml ($P < 0.05$) compared with their respective control values (Fig. 4B).

Effects of sCT and hCG on P450arom gene expression

Total mRNAs were isolated from ovarian follicles treated without or with sCT or hCG (each 50 ng/ml) for different

Figure 4 (A) Time-course effects of sCT and hCG (each 50 ng/ml) on *in vitro* 17 β -estradiol release by the vitellogenic follicles of *C. carpio*. The sCT- and hCG-stimulated conversion of [³H]testosterone to [³H]estradiol-17 β in the ovarian follicles of *C. carpio*. Ovarian follicles were incubated in the presence of [³H]testosterone (1×10^6 c.p.m., 140 mmol) without or with sCT or hCG for 8 h. Each value is the mean $\pm$ s.e.m. of five incubations taking follicles in triplicate from five donor fish (A) * $P < 0.05$ versus saline control (SC) at respective time period, (B) * $P < 0.05$ versus saline control).

time intervals and RT-PCR was performed using P450arom specific primer CYP19A. Figure 5A shows that both hCG and sCT stimulated P450arom gene expression in ovarian follicles incubated for 2 h and increased gradually and significantly ($P < 0.05$) from 4 to 8 h. The expression of GAPDH was used as a loading control (Fig. 5B).

Effects of dbcAMP on sCT-stimulated E₂ release

To evaluate the role of intracellular cAMP in the regulation of sCT-induced E₂ release, effects of dbcAMP (a cAMP analog to mimic increase of intracellular cAMP) on ovarian follicles were examined. Figure 6A shows that dbcAMP at two increasing concentrations (0.5 and 1.0 mM) stimulated the release of E₂ in the medium and addition of sCT (25 ng/ml medium) potentiated the effects of dbcAMP on E₂ release by the follicles.

Effects of adenylate cyclase inhibitor on sCT-stimulated E₂ release

The effects of SQ22536, a cell permeable selective adenylate cyclase inhibitor on sCT- and hCG-stimulated E₂ release by the ovarian follicles, were examined. Administration of SQ22536 at increasing doses (0.1–1.0 mM) attenuated both sCT- and hCG-stimulated E₂ release in the medium in a concentration-dependent manner (Fig. 6B, $P < 0.05$).

Effect of protein kinase C (PKC) inhibitor on sCT-stimulated E₂ production

To ascertain the involvement of PKC in the regulation of E₂ release by sCT, the effect of NPC-15437 dihydrochloride, a selective PKC inhibitor was examined. NPC-15437 at all concentrations tested (0.1–1.0 mM) failed to attenuate sCT-stimulated E₂ release in the medium. However, PKC inhibitor attenuated hCG-stimulated E₂ release in a concentration-dependent manner (Fig. 7, $P < 0.05$).

Discussion

In the present study, we found that administration of sCT to carp *C. carpio* during vitellogenic stage significantly stimulated spontaneous and hCG-induced secretion of E₂ *in vivo* and *in vitro*. We described that ovarian follicles are equipped with CT receptors as evidenced from the specific binding of sCT to the membrane preparation. We reported that sCT stimulated both aromatase activity and P450arom gene expression in the ovarian follicles. Furthermore, we suggested that stimulatory action of sCT on ovarian E₂ secretion was mediated through cAMP pathway.

CT is a hypocalcemic hormone that mineralizes bone by suppressing the activity of osteoclast in mammals

Figure 5 Effect of hCG and sCT on the expression of P450arom gene in ovarian follicles of *C. carpio*. Ovarian follicles were incubated for 2, 4, 6, and 8 h without or with sCT or hCG (each 50 ng/ml). Total RNA was isolated from ovarian follicles and RT-PCR was performed using CYP19A gene specific primer. The amplified product was loaded in agarose gel as control, sCT, and hCG treated samples. (A) The pixel densities of the bands were quantified with ImageJ software National Institute of Health (NIH) and have been represented in bar diagram as relative arbitrary units considering the control value as 1. (B) The expression of GAPDH was used as a loading control. The experiments were performed three times in duplicate, and the values are mean $\pm$ S.E.M., * $P < 0.05$.

(Regnister 1993). In comparison, the role of CT in fish is poorly understood. However, several laboratories have been able to show that CT is indeed capable of altering plasma calcium levels in fish (Wendelar Bonga 1981, Wates & Barrett 1983, Chakrabarti & Mukherjee 1993, Srivastava *et al.* 1998, Mukherjee *et al.* 2004a,b). Nonetheless, inconclusive results are reported from time to time and there is still no consensus as to the role of CT in calcium homeostasis in fish. In contrast, evidence for a physiological role of CT during teleost sexual

maturation is consistent. Histological and ultrastructural studies of ultimobranchial glands of Atlantic salmon, masu salmon, rainbow trout, zebrafish, goldfish, European eel, and Japanese eel all indicate that these glands are maximally active in sexually mature pre-ovulatory females (Oguri 1973, Peignoux-Deville *et al.* 1975, Yamane 1977, 1978, Yamane & Yamada 1977). Plasma CT levels in coho salmon, Japanese eel, and rainbow trout are higher in females during the spawning season and reached a peak just before ovulation

Figure 6 (A) Effects of sCT on *in vitro* release of 17 β -estradiol by the ovarian follicles of *C. carpio* in the absence (hatched bar) or presence of 0.5 mM (cross hatched bar) and 1.0 mM (solid bar) dbcAMP. (B) Effects of SQ22536, a selective inhibitor of adenylate cyclase, on sCT- and hCG-stimulated *in vitro* 17 β -estradiol release by the ovarian follicles of *C. carpio*. Ovarian follicles were incubated in the absence (control) or presence of sCT or hCG with increasing doses of SQ 22536 for 12 h. Each value represents $\pm$ S.E.M. of five incubations taking follicles in triplicate from five donor fish (* P <0.05 versus sCT corresponding control group, * P <0.05 versus hormone alone, + P <0.05 from those shown for tissues incubated with hormone alone).

(Deftos *et al.* 1974, Watts *et al.* 1975, Yamauchi *et al.* 1976, 1978, Bjornsson *et al.* 1986, Norberg *et al.* 1989). The E₂ increases plasma CT levels in rainbow trout (Bjornsson *et al.* 1986, 1989) and a direct induction of estrogen on CT secretion from ultimobranchial glands in goldfish has also been suggested (Suzuki *et al.* 2004). Although a possible explanation for the hyperactivity of ultimobranchial gland and the rise in plasma CT levels during peak reproductive season in female fish has been put forward by many workers (Bjornsson *et al.* 1989, Brown & Bern 1989, Suzuki *et al.* 2004), none of them were able to suggest an exact relationship between CT and reproduction in fish.

The present study provides evidence that sCT is effective in increasing plasma E₂ levels in vitellogenic stage fish and stimulating both spontaneous and hCG-induced secretion of E₂ by the ovarian follicles *in vitro*. The effect of sCT on steroid

production both *in vivo* and *in vitro* was dose- and time-dependent. For our *in vivo* experiment, we used fish kept in high-Ca²⁺ water. As shown previously, the hypocalcemic effects of sCT were less in fish kept in high-Ca²⁺ water than in normal water (Chakrabarti & Mukherjee 1993, Mukherjee *et al.* 2004a,b). In the present study, after sCT injection to vitellogenic fish, the level of plasma Ca²⁺ altered a little but the release of plasma E₂ was significantly increased. We concluded that the increase of plasma E₂ was independent of the Ca²⁺-decreased effect of sCT. Effects of sCT on increased plasma E₂ levels might be due to its action either on high pituitary gonadotropin hormone (GtH) release or its direct action on ovarian follicles. Intravenous infusion of CT in humans caused a calcium-independent reduction in thyrotropin and LH secretion in response to hypothalamic releasing hormone (Leicht *et al.* 1974). Inhibition of sCT on secretion of

Figure 7 Effects of NPC-15437 dihydrochloride, a selective inhibitor of PKC, on (A) sCT- and (B) hCG-stimulated *in vitro* release of 17 β -estradiol by the ovarian follicles of *C. carpio*. Ovarian follicles were incubated in the absence (control) or presence of sCT or hCG with increasing doses of NPC-15437 dihydrochloride for 12 h. Each point represents $\pm$ S.E.M. of five incubations taking follicles in triplicate from five donor fish (* P <0.05 versus hormone alone, + P <0.05 from those shown for tissues incubated with hormone alone).

progesterone and GnRH-stimulated pituitary LH has also been reported (Tsai *et al.* 1999). Moreover, receptors of CT-designated C1a and C1b receptors have been identified in rat brains (Sexton & Hilton 1992, Albrandt *et al.* 1993). All these studies indicate a physiological role for CT at the pituitary and ovarian levels in mammals. However, no such information is yet available in fish. Indeed, in our present study, we did not observe pituitary LH release after sCT injection of fish. Therefore, the possibility of a CT-regulated GtH release by the pituitary cannot be ruled out. As observed in our present study, 25 ng sCT peptides are effective in stimulating spontaneous and hCG-induced *in vitro* E₂ release by the ovarian follicles. Therefore, the reason for the sCT-induced rise in plasma E₂ levels is that sCT stimulated E₂ production by acting directly on ovarian follicular cells in the fish.

A result of the present study shows that sCT can bind specifically to carp ovarian membrane preparation. This indicates the presence of receptor molecules in the carp ovarian follicles which recognize sCT. Binding of CT with membrane preparation was found to be saturable with high affinity ($B_{\max}$, 1.2 pmol/mg protein, K_d 48.8 pmol/l). Available information on the presence of CT-binding sites and sCT-induced inhibition of progesterone secretion in rat granulosa cells indicate a physiological role of CT at ovarian levels in mammals (Tsai *et al.* 1999). Our finding is the first report on the presence of sCT receptor in the fish ovary apart from its presence in gills. Therefore, the presence of functional receptors for CT in the ovarian membrane preparation of vitellogenic follicles and sCT-induced *in vitro* production of E₂ by the ovarian follicles clearly indicate a functional link between binding and specific biological response.

In the present study, significant augmentation of aromatase activity, both in hCG- and sCT-treated ovarian follicles,

is supported by a high rate of conversion of aromatizable androgen (testosterone to E₂) and enhanced the synthesis and release of E₂ under stimulation of both the hormones. We also showed for the first time in teleost that sCT stimulated P450arom gene expression in the ovarian follicles. It has been well documented that the fish ovarian follicles possess an aromatase enzyme participating in the conversion of aromatizable androgen to E₂, while P450arom mRNA levels, are increased in association with the increase of enzyme activity under the stimulation of hCG (Gen *et al.* 2001, Kagawa *et al.* 2003).

It has been well established that in the fish ovary, gonadotropin stimulates steroid production involving both PKA and PKC pathways (Nagahama 1987, Srivastava & Van der Kraak 1994). In our experiment, we found that in addition to the lone effect of sCT on E₂ production, stimulatory effects of hCG on E₂ production *in vitro* were potentiated in the presence of sCT. Results indicate that administration of dbcAMP stimulated sCT-induced E₂ production and cell permeable selective inhibitor of adenylate cyclase, SQ 22356 attenuated both hCG- and sCT-induced E₂ production. The specific PKC inhibitor NPC-15437 dihydrochloride on the other hand had no inhibitory effects on sCT-stimulated E₂ release. Therefore, we suggest that the signal for sCT-stimulated E₂ release might be transduced through the cAMP pathway and interaction between sCT and hCG on the signal transduction in the fish ovary is still open to question. Increased production of cAMP caused by CT that has been demonstrated in perfused rat bone, osteoblast-like cell line, osteoclast, atria and aortic smooth muscle (Kubota *et al.* 1985, Sugimoto *et al.* 1986, Nicholson *et al.* 1987, Wang & Fiscus 1989, Iida-Klein *et al.* 1992), and in the rat testicular and anterior pituitary gland (Wang *et al.* 1994) substantiate

the action of CT through the cAMP pathway in the fish ovarian follicles.

Our present finding on the stimulatory role of sCT on basal and hCG-induced E₂ production by fish ovarian follicles is completely opposite to the observed action of CT in the mammalian ovary and testes in the regulation of steroid production. Our unpublished data with ovarian follicles of perch *Anabas testudineus* also showed the same stimulatory action of sCT on E₂ production. Although the action of CT is well characterized in mammals, its action in fish, particularly with regard to calcium regulation is still controversial. Therefore, the observed stimulatory effect of CT on fish ovarian steroidogenesis, in contrast to mammals, is not unusual. It is most likely that CT has evolved a distinct function in different lineage, which probably relates to its aquatic life and this needs further studies on higher group of vertebrates. In addition, the exact physiological relevance of the stimulation of E₂ production by sCT in the fish ovary, when GtH-stimulated E₂ production is normally operative, is not clear. Researchers have become increasingly aware that the traditional concept of the action of GtH in the regulation of ovarian growth, maturation, and steroidogenesis may no longer be tenable. Localization of several neuropeptides in the nerve that innervate the ovary, neuropeptide Y, substance P, vasoactive intestinal polypeptide (VIP) and somatostatin in the ovary of mammals have already been reported (Ojeda *et al.* 1985, Ahmed *et al.* 1986, McDonald *et al.* 1987). Although the function of most of these peptides in the ovary remain unknown, stimulatory effects of VIP on estrogen and progesterone release from cultured granulosa cells have been reported. Reports are also available that the stimulatory action of VIP appears to be exerted, at least in part, through a direct stimulatory action of neuropeptides on the synthesis of the cholesterol-side chain cleavage enzyme (Trzeciak *et al.* 1986, 1987). Recently, Clark *et al.* (2002) reported for the expression of the CT gene in the ovary of a teleost, *Fugu rubripes* and suggested that CT may act as a potential neuropeptide. Considering all these, it would appear that CT in fish may take some role, at least in part, to support the action of GtH on the ovary during vitellogenic growth by acting independently or synergistically with GtH. This assumption supports the high plasma CT levels in fish during this phase of gonadal growth (Deftos *et al.* 1974, Yamauchi *et al.* 1978, Bjorsson *et al.* 1986).

In summary, the present findings suggest that sCT stimulates E₂ production in vitellogenic ovarian follicles of carp *C. carpio* by acting directly on the ovary, without altering plasma calcium levels. Membranes of ovarian follicles are equipped with functional CT receptors with high affinity. sCT could stimulate both basal and hCG-stimulated E₂ release. The stimulatory effect of sCT on E₂ production is associated with an increase of P450 aromatase activity and P450arom gene expression in ovarian follicles. The signal transduction of the stimulatory effects of sCT is mediated through cAMP pathway.

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