Localization of gonadotrophin-releasing hormone I, bradykinin and their receptors in the ovaries of non-mammalian vertebrates

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Abstract

GnRH I and its receptors have been demonstrated in the ovaries of various vertebrates, but their physiological significance in reproductive cascade is fragmentary. Bradykinin is a potent GnRH stimulator in the hypothalamus. In the present study, the presence of GnRH I and its receptor, and bradykinin and its receptor in the ovaries of non-mammalian vertebrates were investigated to understand their physiological significance. GnRH I immunoreactivity in the ovaries of fish, frog, reptile and bird were mainly found in the oocyte of early growing follicles and granulosa cells and theca cells of previtellogenic follicles. Vitellogenic follicles showed mild GnRH immunoreactivity. GnRH I-receptor and bradykinin were localized in the same cell types of the ovaries of these vertebrates. The presence of GnRH I, GnRH I-receptor and bradykinin in the ovaries of these vertebrates was confirmed by immunoblotting. The presence of GnRH I mRNA was demonstrated in the ovary of vertebrates using RT-PCR. The ovaries of reptiles and birds showed significantly higher intensity of immunoreactivity for GnRH I-receptor as compared with the fish and amphibian. This may have a correlation with the higher yolk content in the ovary of reptile and bird. These results suggest the possibility of GnRH I and bradykinin as important regulators of follicular development and vitellogenesis in the vertebrate ovary.

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Introduction

The hypothalamic gonadotrophin-releasing hormone (GnRH) is the key regulator of reproductive functions in all the vertebrate species. The major function of this decapeptide is to modulate the synthesis and release of gonadotrophins from the pituitary. GnRH binds to its receptor localized on the pituitary gonadotrophs, where it is involved in the synthesis and release of gonadotrophins. The gonadotrophins in turn stimulate hormone and gamete production by the testes and ovaries. Now, it is well documented that GnRH is not exclusively synthesized in the hypothalamus. Several *in vivo* (Sakamoto *et al.* 1993, Srivastava *et al.* 1995, Raga *et al.* 1998) and *in vitro* studies have shown that GnRH ligands and their agonists can have various extra-hypothalamic functions (Ortmann & Diedrich 1999). A number of studies have shown that GnRH and its receptor are also expressed in some peripheral reproductive tissues including mammary gland, ovary, endometrium and placenta, suggesting that the peptide hormone may have other extrapituitary functions in addition to its role as a GnRH (Harrison *et al.* 2004).

The intra-ovarian production of GnRH has been reinforced by demonstration of ovarian compounds

with GnRH-like activity (Aten *et al.* 1986, 1987) and ovarian *GnRH* gene expression (Peng *et al.* 1994). The presence of GnRH-binding sites in ovarian tissues suggests that GnRH acts through receptor-mediated processes in the ovary (Schirman-Hildesheim *et al.* 2005). In the mammalian ovary, GnRH I and its type-I receptors (GnRH I-receptor) are localized in the granulosa cells (GCs; Jones & Hsueh 1980, Reeves *et al.* 1982, Kang *et al.* 2003) and luteal cells (Clayton *et al.* 1979, Bauer-Dantoin & Jameson 1995), suggesting that this peptide may have autocrine and/or paracrine regulatory function in the ovary. Importantly, unlike the pituitary, ovarian GnRH I-receptors are activated by locally produced GnRH I, because the half-life of this peptide is only a few minutes, and they are found in the peripheral circulation in undetectable concentrations. A myriad of functions have been attributed to GnRH in the mammalian ovary (for review, Leung *et al.* 2003), such as a role in oocyte maturation (Hillensjo & LeMaire 1980), follicular selection and apoptosis, steroidogenesis and effects on the corpus luteum (Srivastava *et al.* 1995, Kang *et al.* 2003). The expression of GnRH in immature gonads suggests that the role of GnRH is considerably broader than that understood presently.

The factor(s) regulating GnRH I synthesis and secretion in the ovary remains poorly understood. Bradykinin emerged as a potent GnRH stimulator from hypothalamic fragment and bradykinin has been shown to localize in the hypothalamic area (Shi *et al.* 1998, 1999). The fact that bradykinin neurons in the hypothalamus play a physiological role in the control of GnRH and luteinizing hormone (LH) release is supported by the finding that central administration of the bradykinin B₂ receptor antagonist into the third cerebral ventricle blocked the steroid-induced LH surge in the ovariectomized adult female rat. This suggests that bradykinin acts directly on GnRH neurons through a mechanism involving mediation by the bradykinin B₂ receptor. It has already been reported that kinin-producing activity increases during ovulation (Smith & Perks 1983, Espey *et al.* 1989, Gao *et al.* 1992). In a study, Kihara *et al.* (2000) showed the presence of a component of bradykinin and bradykinin-producing system in the porcine ovarian follicle, suggesting its role in early follicular development and ovulation. It has been demonstrated that bradykinin induces ovulation in perfused rabbit ovaries (Yoshimura *et al.* 1988, Hellberg *et al.* 1991), potentiates the action of LH (Brännström & Hellberg 1989) and a physiological role of bradykinin in the LH surge was also implicated (Shi *et al.* 1998). Whether bradykinin is also involved in the regulation of ovarian GnRH I synthesis and secretion requires investigation. Therefore, in the present study, the localization and distribution of GnRH I and bradykinin and their receptors were investigated in the ovary of non-mammalian vertebrates (fish, amphibia, reptiles and aves).

Materials and Methods

Sample collection

All experiments were conducted in accordance with the principals and procedures approved by the Departmental Research Committee at Banaras Hindu University, Varanasi, India. Adult females, four to six in number and belonging to different vertebrate classes, were captured in their reproductively active phase from Varanasi and adjacent areas; for example, fish, *Heteropneustes fossilis* (between February and September); amphibia, *Rana tigrina* (between June and July); reptile, *Calotes versicolor* (between March and September) and aves, *Estrilda amandava* (between August and September). Animals were killed by decapitation as soon as they were taken to the laboratory. Ovaries were dissected out. One side of the ovary from each animal was cleaned and kept either frozen at -20 °C for immunoblot analysis or in the RNA later (Ambion, Austin, TX, USA) at -20 °C for RNA extraction and real-time PCR analysis respectively. The ovary from the other side was used for immunohistochemistry. Each ovary was fixed in Bouin's fluid for 24 h, dehydrated in ethanol, cleared in xylene, embedded in paraffin wax and serially sectioned at 6 µm.

Classification of ovarian follicles/oocytes of non-mammalian vertebrates

The ovarian follicles/oocytes are classified into the following types based on their size, presence of vacuoles in the periphery and amount and types of yolk accumulation: a) primordial/primary follicle – oocyte small with translucent cytoplasm, GCs and theca cells not present; b) secondary previtellogenic follicle – oocyte medium-sized with opaque cytoplasm and showing varying amount of peripheral vacuoles, GCs present; c) secondary vitellogenic follicle – medium-sized oocytes with opaque cytoplasm containing pigmented granules and both granulosa and theca cells present and d) tertiary fully grown follicle – large oocytes with cytoplasm containing large yolk platelets, several layers of granulosa and thecal cells (TCs) present.

RNA purification

The total RNA from the ovary was isolated using Agilent total RNA Isolation Mini kit (Product no. 5185-6000; Agilent Technologies Inc., Wilmington, DE, USA). The purity and concentration of the isolated total RNA was checked in Agilent 2100 Bioanalyzer (Functional Genomics Core Facility, Morehouse School of Medicine, Atlanta, GA, USA).

RT reaction

The total RNA from each ovarian sample (2 µg) was converted to cDNA by RT reaction using RT Taqman Kit (Part no: 808-0234; Applied Biosystem Inc., Foster City, CA, USA). Hypothalamic RNA sample was used as control and reverse transcribed along with the ovarian RNA. The reaction protocol was followed according to the manufacturer's instructions. The reaction mixture contained 1 × RT buffer, 5.5 mM MgCl₂, 500 µM/dNTP, 2.5 µM random hexamer, 0.4 U/µl RNase inhibitor and 3.125 U/µl MultiScribe Reverse Transcriptase in final volume of 100 µl. RT reactions were carried out in a DNA Thermal cycler using a program with the following reaction condition: activation of enzyme at 25 °C for 10 min, cDNA formation at 48 °C for 37 min and finally incubation at 95 °C for 5 min to inactivate the enzyme and denature RNA–DNA hybrid. The quality of ssDNA and RNA was checked spectrophotometrically by taking absorbance at 260 and 280 nm.

Relative real-time PCR

Sequence of GnRH I mRNA (rat and human) were obtained from the GenBank database of the National Center for Biotechnology Information of NIH, USA (<http://www.ncbi.nlm.nih.gov/Genbank/index.html>). Using OligoPerfect Designer software (Invitrogen) primer and probe sequences were selected to optimally

Table 1 Details of primers used in the RT-PCR.

Gene	Reference	Primers sequence (5' to 3')	Length of primer	Annealing temperature
<i>GnRH I</i>	Rat GnRH I mRNA	L: AGGAGGATC AATGGCAGAACC	22	62.67
	Accession	R: TCTTCAATCAGACGTTCCAGAGC	23	62.77
<i>GnRH I</i>	Human GnRH I, mRNA	L: CTA CTG ACT TCG TGC GTG GA	20	45
	Accession NM 000825.2	R: CTG CCA GTT GAC CAA CCT CT	20	45
<i>GnRH I</i>	Rat GnRH I R mRNA	L: CTAACAATGCGTCTCTTGA	19	52.28
	Accession	R: TCCAGATAAGGTTAGAGTCG	20	52.09
<i>GnRH I</i>	Human GnRH I R mRNA	L: TTG CCT TTT TAA ACC CAT GC	20	53
	Accession	R: AAC ATG CTC CAA CAT TTG TG	20	53
18S rRNA	Rat 18 S rRNA	L: AAT TCC GAT AAC GAA CGA GA	20	56.3
		R: ATC TAA GGG CAT CAC AGA CC	20	60.4

hybridize and amplify target cDNA sequence for real-time PCR assay. To avoid amplification of contamination of genomic DNA, primers specific for both GnRH I and 18S (housekeeping gene) were designed for cross-exon/intron boundaries. The primer sequences of GnRH I and 18S are described in Table 1.

The real-time PCR assay was performed in an iCycler iQ Real time PCR detection system (Bio-Rad Laboratories) using cDNA (750 or 1125 ng) for each reaction. Each gene expression was assessed in a separate PCR and each reaction mixture consisted of 2 µl left and right primers (0.35 nM/µl concentration) of GnRH I and 18S respectively, 10 µl of 2 × quantiTech SYBERGreen PCR buffer, dNTP mix including dUTP, fluorescent dyes like SYBERGreen1 and ROX and 5 mM MgCl₂ procured from Qiagen and DNase–RNase-free water to make a total volume of 20 µl. Samples were amplified with a pre-cycling hold of 95 °C for 15 min followed by 50 cycles of denaturation at 94 °C for 15 s, annealing at 54 °C for 30 s and extension at 72 °C for 30 s. The reaction was terminated at 72 °C for 5 min and cooled to 4 °C. Quantification results were expressed in terms of cycle threshold value (Table 2).

Antibodies

All the antisera used in this study were obtained from commercial sources (Table 3). GnRH I antibody used in this study was highly specific and showed only 6.6% cross-reactivity with lamprey GnRH, but very minimal cross-reactivity with other GnRHs including chicken GnRH (GnRH II) and salmon GnRH (GnRH III) based on

assessments by the manufacturer using sensitive RIA applications. A highly specific GnRH I-receptor antibody used was an MAB against 1-29 amino acid epitope at the amino terminus of human GnRH I-receptor.

Immunohistochemistry

Ovarian sections were processed through a standard protocol of immunohistochemistry (Table 3). After deparaffinization and rehydration, endogenous peroxidase was quenched with 0.3% H₂O₂, equilibrated in 0.05 mol/l Tris–Cl–0.15 mol/l NaCl (TBS, pH 7.3). Background blocking was performed with normal horse serum. The tissue sections were incubated for 1 h at room temperature with rabbit polyclonal antibody against human GnRH I (GnRH I, Peninsula Lab Inc., San Carlos, CA, USA) diluted to 1:1500, GnRH I-receptor (Santacruz Biotechnology Inc., Santa Cruz, CA, USA) antibody diluted to 1:25 and bradykinin (Peninsula Lab Inc.) antibody diluted to 1:2000 and bradykinin B₂ receptor (BD Transduction Laboratories, San Jose, CA, USA) antibody diluted to 1:25 in TBS. The detection system used was ABC staining kit from Vector Laboratories, Novo Castra (UK). The peroxidase activity was revealed in 0.03% 3,3'-diaminobenzidine tetrahydrochloride (DAB; Sigma) in 0.01 M Tris–Cl (pH 7.6) and 0.1% H₂O₂. Nucleus was counterstained with Elrich's hematoxylin. For preabsorbed control, the primary antibodies (in the same dilution as used for localization) were incubated with the pure antigen in the final concentration of 20 ng/100 µl overnight at 4 °C.

Table 2 Relative expression ratio of mRNA expression of gonadotrophin-releasing hormone (GnRH) and GnRH I-receptor by real-time PCR in the vertebrate ovary.

Species	Tissue	Rat primers		Human primers	
		GnRH I	GnRH I receptor	GnRH I	GnRH I receptor
Fish	Ovary	NA	NA	26.91	NA
Amphibia	Ovary	0.19	NA	20.25	NA
Reptile	Ovary	5.04	NA	0.52	NA
Aves	Ovary	0.05	NA	NA	NA

The reference gene used is 18S rRNA and expression compared with the control, which is hypothalamus in case of GnRH I and pituitary in the case of GnRH I-receptor.

Table 3 Details of antibodies used for immunohistochemistry and western/slot blot.

Antibody	Type	Source	Concentration used for IHC	Concentration used for western/slot blot	Control
GnRH I	GnRH I	Peninsula Lab Inc, San Carlos, CA, USA (Cat No. IHC- 7201)	1:1500	1:2000	Preabsorbed negative control
GnRH I receptor	GnRH I-R	Santa Cruz Biotechnology Inc. (Cat no. sc 8028)	1:25	1:250	Negative
Bradykinin	Bradykinin	Peninsula Lab Inc, San Carlos, CA, USA (Cat no. IHC 7051)	1:2000	1:2000	Preabsorbed negative control
Bradykinin receptor	BK-R	BD Transduction Lab, CA, USA (Cat no. 610451)	1:25	1:250	Negative

Slot blot

Protein was extracted as described elsewhere (Chanda *et al.* 2004). Equal amount of protein as determined by Folin's method was equated with PBS. Ten micrograms of this sample were loaded on nitrocellulose (NC) membrane using Millipore slot blot apparatus. Non-specific sites were blocked with 5% non-fat-dried milk in TBS, 0.02% Tween 20. The membrane was then incubated with rabbit anti-human GnRH I antibody (1:2000) or rabbit anti-human bradykinin antibody (1:2000). Immunodetection was performed with anti-rabbit immunoglobulin G horseradish peroxidase (IgG-HRP) conjugated (1:1000). Finally, membrane was developed with ECL. Validation of the slot blot assay was performed using serially diluted ovarian protein samples ranging between 2 and 20 µg/µl. The intensity of the protein bands of the slot blot was quantified using densitometry. The graph plotted between the amounts of protein loaded and the intensity of the protein band showed strong correlation ($r=0.98$; Fig. 1). The intra-assay coefficient of variation was <7.5% and the sensitivity of the blot was ~2 µg/µl.

Western blot

Protein was extracted as described elsewhere (Chanda *et al.* 2004). Equal amount of proteins (300 µg) as

Figure 1 Validation of slot-blot assay. The amounts of protein loaded and intensity of the protein band in the slot blot showing strong correlation ($r=0.98$).

determined by Folin's method was loaded on SDS-PAGE for electrophoresis. The protein was transferred on nitrocellulose membrane (Sigma). Non-specific sites were blocked with 5% non-fat-dried milk in TBS, 0.02% Tween 20. The membrane was then incubated with mouse anti-human GnRH I-receptor antibody (1:250) or mouse anti-human bradykinin receptor antibody (1:250). Immunodetection was performed with anti-mouse IgG-HRP conjugated (1:1000). Finally, membrane was developed with DAB. Blot was repeated thrice for each protein.

Densitometry

The densitometric analysis was performed by scanning and subsequently quantifying the blots using a computer-assisted image-analysis system (AlphaEaseFC software, Alpha Innotech Corporation, San Leandro, CA, USA). The system was calibrated to have constant parameter for light (intensity and area of light beam) throughout the experiment. Absorbance was expressed as integrated density value. Each measurement was repeated twice. The data were presented as the mean of the integrity density value $\pm$ S.E.M. of three blots of each peptide.

Results

Presence of GnRH I mRNA in the ovaries of different species of non-mammalian vertebrate

The mRNA for GnRH I has been found using primers for rat and human *GnRH* gene in the ovaries of various vertebrates and their relative Ct values in comparison with mammalian hypothalamus (Table 1).

Distribution of GnRH I and GnRH I-receptor in the ovaries of different species of non-mammalian vertebrate

Ovarian sections of fish, frog, reptile and bird during their reproductively active period were immunostained for GnRH I and GnRH I-receptor to demonstrate their localization and the results are summarized in Tables 4–7 and Figs 2–5.

Table 4 Intensity and distribution of gonadotrophin-releasing hormone (GnRH), bradykinin and their receptors immunoreactivity in the ovary of fish.

Type of follicles	Intensity			
	GnRH	GnRH receptor	Bradykinin	Bradykinin receptor
Primary follicle				
Oocyte	—/+	—/+	—/+	—/+
Granulosa	*	*	*	*
Theca	*	*	*	*
Secondary previtellogenic follicle				
Oocyte	+++	+++	+++	+++
Granulosa	+++	+	+++	++
Theca	++	+	+	+
Secondary vitellogenic follicle				
Oocyte	+++	++	++	++
Granulosa	+	+	+	+
Theca	+	+	+	+
Tertiary follicle				
Oocyte	—	—	—	—
Granulosa	—	—	—	—
Theca	—	—	—	—
Stroma	*	*	*	*
Preabsorbed control	—		—	

Scores for intensity of immunoreactivity are as follows: —, absence of immunoreactivity; +, mild; ++, moderate; +++, intense; *, absence of the cells in the section.

The primordial and primary follicles generally showed no immunoreactivity in all the vertebrate species. GnRH I immunoreactivity appeared first in the oocyte of early previtellogenic secondary follicles. GnRH I immunoreactivity was much stronger in the oocytes of fish and frog than in the oocytes of reptile and bird. In ovarian follicles of bird and reptile, GnRH I immunoreactivity is localized mainly in GCs and TCs, whereas oocyte shows mild or no immunoreactivity. The intensity of GnRH I immunoreactivity increases as the follicles increase in size and immunoreactivity localized in the periphery of oocytes, GCs and TCs. The maximum

intensity of GnRH I immunoreactivity was found in the early vitellogenic follicles. The GnRH I immunoreactivity declined to mild or no staining in the fully grown vitellogenic tertiary follicles. Atretic follicles also showed no immunoreactivity. In the ovary of reptile and bird, GnRH I immunoreactivity was also seen in the interstitial cells. The pattern of GnRH I-receptor immunoreactivity in the ovaries of fish, frog, reptile and bird was nearly the same as described for GnRH I. The maximum GnRH I-receptor immunoreactivity was found in the previtellogenic and vitellogenic secondary follicles. Immunoreactivity was seen in the oocytes and

Table 5 Intensity and distribution of gonadotrophin-releasing hormone (GnRH), bradykinin and their receptors immunoreactivity in the ovary of amphibia.

Type of follicles	Intensity			
	GnRH	GnRH receptor	Bradykinin	Bradykinin receptor
Primary follicle				
Oocyte	+	+	+	+
Granulosa	*	*	*	*
Theca	*	*	*	*
Secondary previtellogenic follicle				
Oocyte	++	++	+++	+++
Granulosa	++	++	++	++
Theca	+	+	+	+
Secondary vitellogenic follicle				
Oocyte	++	++	++	++
Granulosa	++	+	+	+
Theca	+	++	++	++
Tertiary follicle				
Oocyte	—	—	—	—
Granulosa	—	—	—	—
Theca	—	—	—	—
Stroma	*	*	*	*
Preabsorbed control	—		—	

Scores for intensity of immunoreactivity are as follows: —, absence of immunoreactivity; +, mild; ++, moderate; +++, intense; *, absence of the cells in the section.

Table 6 Intensity and distribution of gonadotrophin-releasing hormone (GnRH), bradykinin and their receptors immunoreactivity in the ovary of reptile.

Type of follicles	Intensity			
	GnRH	GnRH receptor	Bradykinin	Bradykinin receptor
Primary follicle				
Oocyte	—	—	—	—
Granulosa	+	+	+	+
Theca	*	*	*	*
Secondary previtellogenic follicle				
Oocyte	++	++	++	++
Granulosa	+++	+++	+++	+++
Theca	+	+	+	+
Secondary vitellogenic follicle				
Oocyte	++	++	++	++
Granulosa	+++	+++	+++	+++
Theca	++	++	++	++
Tertiary follicle				
Oocyte	+	+	+	+
Granulosa	+	+	+	+
Theca	+	+	+	+
Stroma	—	—	—	—
Preabsorbed control	—	—	—	—

Scores for intensity of immunoreactivity are as follows: —, absence of immunoreactivity; +, mild; ++, moderate; +++, intense; *, absence of the cells in the section.

GCs in fish and frog, whereas in the GCs and TCs in the reptile and bird follicles.

Distribution of bradykinin and bradykinin B₂ receptor (BK-R) in the ovaries of different species of non-mammalian vertebrates

Ovarian sections of fish, frog, reptile and bird during their reproductively active period were immunostained for bradykinin and BK-R to demonstrate their localization and results are summarized in [Tables 4–7](#) and [Figs 2–5](#).

The primordial and primary follicles generally showed no immunoreactivity for bradykinin and BK-R in all the vertebrate species. Moderate to intense bradykinin immunoreactivity was noticed in the oocyte, GCs and TCs of previtellogenic and vitellogenic secondary follicles, whereas fully grown vitellogenic tertiary follicles showed mild to no immunoreactivity in the ovaries of fish, frog, reptile and bird. A strong immunoreactivity was also noticed in the interstitial cells of the reptile and bird ovaries. The pattern of BK-R immunoreactivity in the ovaries of fish, frog, reptile and bird was nearly the same as

Table 7 Intensity and distribution of gonadotrophin-releasing hormone (GnRH), bradykinin and their receptors immunoreactivity in the ovary of bird.

Type of follicles	Intensity			
	GnRH	GnRH receptor	Bradykinin	Bradykinin receptor
Primary follicle				
Oocyte	—	—	—	—
Granulosa	+	++	++	++
Theca	*	*	*	*
Secondary previtellogenic follicle				
Oocyte	+	+	+	+
Granulosa	+++	+++	+++	+++
Theca	++	+	++	+
Secondary vitellogenic follicle				
Oocyte	—	—	—	—
Granulosa	+++	+++	+++	+++
Theca	++	++	++	++
Tertiary follicle				
Oocyte	—	—	—	—
Granulosa	++	++	++	++
Theca	+	+	+	+
Stroma	—	—	—	—
Preabsorbed control	—	—	—	—

Scores for intensity of immunoreactivity are as follows: —, absence of immunoreactivity; +, mild; ++, moderate; +++, intense; *, absence of the cells in the section.

Figure 2 All figures are transverse sections of the ovary of fish, *Heteropneustes fossilis*. (A) Ovary showing GnRH I immunoreactivity mainly in the oocyte of growing follicles. Previtellogenic follicle shows mild GnRH I immunoreactivity throughout the cytoplasm of oocyte. Early vitellogenic follicle shows immunoreactivity in the periphery of oocyte, whereas late vitellogenic follicle shows little or no immunoreactivity. The primary follicle shows no immunoreactivity. (B) Early vitellogenic follicle enlarged to show GnRH I immunoreactivity in the periphery of oocyte. (C) Late vitellogenic follicle shows little or no immunoreactivity. (D) Preabsorbed control ovarian section showing no GnRH I immunostaining in the oocyte of secondary follicles. (E) Ovary showing GnRH I-receptor immunoreactivity in the oocyte of secondary follicles. (F) Early vitellogenic follicle showing GnRH I-receptor immunoreactivity in the periphery of oocyte. (G) Early vitellogenic follicle enlarged to show GnRH I-receptor immunoreactivity in the periphery of oocyte. (H) Ovary showing bradykinin immunoreactivity mainly in the oocyte of growing follicles. Previtellogenic follicle shows mild bradykinin immunoreactivity throughout the cytoplasm of the oocyte. Early vitellogenic follicle shows immunoreactivity in the periphery of the oocyte, whereas late vitellogenic follicle shows little or no immunoreactivity. (I) Early vitellogenic follicle showing bradykinin immunoreactivity in the periphery of the oocyte. (J) Preabsorbed control ovarian section showing no bradykinin immunostaining in the oocyte of secondary follicles. (K) Ovary shows bradykinin receptor immunoreactivity in the oocyte of secondary follicles. (L) Early vitellogenic follicle shows bradykinin receptor immunoreactivity in the periphery of the oocyte. (M) Early vitellogenic follicle enlarged to show bradykinin receptor immunoreactivity in the periphery of the oocyte.

described for bradykinin. The maximum BK-R immunoreactivity was found in the previtellogenic and vitellogenic secondary follicles. Immunoreactivity was seen in the oocytes and GCs in fish and frog, whereas in the GCs and TCs in the reptile and bird follicles.

Relative concentration of GnRH I, bradykinin and their receptors in the ovaries of different species of non-mammalian vertebrates

GnRH I and bradykinin in the ovary and hypothalamus of fish, frog, reptile and bird were detected by slot blot. The variations in the intensity of immunostaining of the slot blots representing the concentration of GnRH I/bradykinin were measured by densitometry and results are shown in Figs 6 and 7. The densitometric analysis of GnRH I and bradykinin slot blots showed marked variation in the immunoreactivity between the ovaries of different vertebrate species. The ovary of the reptile showed distinctly higher intensity of GnRH I and

bradykinin immunostaining when compared with the ovaries of other vertebrates. The hypothalamus of frog showed a distinctly higher intensity of GnRH I immunostaining when compared with the hypothalamus of other vertebrates.

Western blot analysis of GnRH I-receptor in the ovaries of fish, frog, reptile and bird showed a single immunoreactive band at 62 kDa. Densitometric analysis of western blot of GnRH I-receptor in the ovaries of different vertebrate species showed a marked variation. The ovaries of reptile and bird showed significantly higher intensity of immunoreactivity of GnRH I-receptor when compared with the ovaries of fish and frog as well as with the pituitary of rat.

Western blot analysis of BK-R in the ovary of fish, frog, reptile and bird showed two immunoreactive bands at 42 and 44 kDa respectively. Densitometric analysis of western blot of BK-R in the ovaries of different vertebrate species showed a marked variation with the highest immunoreactivity found in the bird ovary.

Immunohistochemistry in Amphibia, *Rana tigerina*

Figure 3 All figures are the transverse section of the ovary of the amphibia, *Rana tigerina*. (A) Ovary showing GnRH I immunoreactivity mainly in the oocyte of growing follicles. Previtellogenic follicle shows mild GnRH I immunoreactivity throughout the cytoplasm of oocyte. Late vitellogenic follicle shows little or no immunoreactivity. The primary follicle shows no immunoreactivity. (B) Early vitellogenic follicle enlarged to show GnRH I immunoreactivity in the periphery of oocyte. (C) Preabsorbed control ovarian section showing no GnRH I immunostaining in the oocyte of secondary follicles. (D) Ovary shows GnRH I-receptor immunoreactivity in the oocyte of secondary follicles. (E) Early vitellogenic follicle shows GnRH I-receptor immunoreactivity in the periphery of oocyte. (F) Early vitellogenic follicle enlarged to show GnRH-receptor immunoreactivity in the periphery of oocyte. (G) Ovary showing bradykinin immunoreactivity mainly in the oocyte of growing follicles. Previtellogenic follicle shows mild bradykinin immunoreactivity throughout the cytoplasm of oocyte. Early vitellogenic follicle shows immunoreactivity in the periphery of oocyte, whereas late vitellogenic follicle shows little or no immunoreactivity. (H) Early vitellogenic follicle shows bradykinin immunoreactivity in the periphery of oocyte. (I) Preabsorbed control ovarian section showing no bradykinin immunostaining in the oocyte of secondary follicles. (J) Ovary shows bradykinin receptor immunoreactivity in the oocyte of secondary follicles. (K) Early vitellogenic follicle shows bradykinin receptor immunoreactivity in the periphery of oocyte. (L) Early vitellogenic follicle enlarged to show bradykinin receptor immunoreactivity in the periphery of oocyte.

Discussion

The present immunocytochemical study demonstrates, for the first time, the distribution of GnRH I, bradykinin and their receptors in the ovaries of fish, frog, reptile and bird during the reproductively active phase. The presence of GnRH I was confirmed by the demonstration of its mRNA in the ovaries of fish, frog, reptile and bird using mammalian primers. The presence of GnRH I, bradykinin and their receptors was further confirmed by slot/western blot. Bradykinin has been suggested to be a potent stimulator of GnRH I release in the hypothalamus (Shi *et al.* 1998); however, whether bradykinin causes release of GnRH I in the ovary is not known. The presence of bradykinin together with GnRH I in the ovarian cell suggests the possibility of bradykinin as a regulator of GnRH release. The presence of complete GnRH I system with the ligand peptide and mRNA, and receptor and its regulator existing within the ovaries of fish to bird thus support an intra-ovarian role for GnRH I peptide in the vertebrate ovaries, which appears to be conserved during evolution.

GnRH has often been demonstrated in the ovary of several mammalian as well as non-mammalian vertebrates using RT-PCR techniques without functional assays of protein expression and localization. In non-mammalian vertebrates, GnRH I peptide has been isolated and biochemically characterized from the ovary of goldfish (Pati & Habibi 1998, Yu *et al.* 1998). In the ovary of the frog, *Rana esculenta*, GnRH-like substance has been identified using high performance liquid chromatography followed by RIA (Battisti *et al.* 1994), whereas GnRH immunoreactivity using cGnRH II antiserum is observed in the ovary of newt, *Triturus carnifex* (Battisti *et al.* 1997). However, immunoreactive GnRH could not be detected in the ovary of lungfish, *Protopterus annectens* (King *et al.* 1995). In the present study, the presence of both mRNA and peptide for GnRH I are demonstrated in the ovary of vertebrate species of fish, amphibia, reptilia and birds. The presence of mRNA is shown by RT-PCR, whereas GnRH I peptide is demonstrated by immunocytochemistry (ICC) and slot blot using human GnRH I antiserum in the ovaries of

Figure 4 All figures are the transverse sections of the ovary of the reptile, *Calotes versicolor*. (A) Ovary showing GnRH I immunoreactivity mainly in the small granulosa cell located towards the oocyte of growing follicles. (B) Early vitellogenic follicle enlarged to show GnRH I immunoreactivity in the periphery of oocyte. (C) Preabsorbed control ovarian section showing no GnRH I immunostaining in the granulosa cells as well as in the oocyte of secondary follicle. (D) Ovary shows GnRH I-receptor immunoreactivity in the oocyte of secondary follicles. (E) Early vitellogenic follicle shows GnRH I-receptor immunoreactivity in the small granulosa cells. (F) Early vitellogenic follicle shows GnRH I-receptor immunoreactivity in the granulosa cells and late vitellogenic follicle shows GnRH I-receptor immunoreactivity in the periphery of oocyte. (G) Ovary showing bradykinin immunoreactivity mainly in the smaller granulosa cells and periphery of the oocyte of growing follicles. (H) Early vitellogenic follicle shows bradykinin immunoreactivity in the periphery of oocyte. (I) Preabsorbed control ovarian section showing no bradykinin immunostaining in the oocyte of secondary follicles. (J) Ovary shows bradykinin receptor immunoreactivity in the granulosa cells of secondary follicles. (K) Early vitellogenic follicle shows bradykinin receptor immunoreactivity in the small granulosa cells and periphery of oocyte. (L) Early vitellonic follicle enlarged to show bradykinin receptor immunoreactivity in the thecal cells.

vertebrate species of fish, amphibia, reptilia and bird. Within the ovary, GnRH I is localized mainly in the growing follicles. In previtellogenic follicles, mild GnRH I immunoreactivity is mainly found in the outer margin of oocytes. In the vitellogenic follicles, GnRH I immunoreactivity is observed in the GCs and TCs. The presence of GnRH I and its receptors in the GCs and TCs suggest that GnRH I exerts its action in an autocrine/paracrine manner (Peng *et al.* 1994). GnRH is an intra-ovarian regulatory factor supported by localization of GnRH I and its receptors in the ovarian tissues as well as by previous observations on the direct effects of this decapeptide on folliculogenesis and steroidogenesis (Stojilkovic *et al.* 1994, Andreu *et al.* 1998, Kang *et al.* 2000, Irusta *et al.* 2003). The shift in immunoreactivity from oocyte to GCs and TCs as follicles grow is intriguing and needs further investigation. The present study also showed an increase in the GnRH I immunoreactivity in the GCs and TCs as the follicles grow and the absence of GnRH I immunoreactivity in atretic follicles. These observations suggest that GnRH I promotes follicular development in the vertebrate ovary. This is consistent with a recent study suggesting the proliferative role of

GnRH in the pituitary gonadotropes (Miles *et al.* 2004). The *in situ* studies on the rat ovary have indicated the presence of the GnRH I-receptor mRNA in the GCs of the primary, secondary and tertiary follicles (Kogo *et al.* 1999).

The presence of GnRH-like peptides was first identified in luteinized rat ovaries (Aten *et al.* 1986), and the expression of GnRH has been localized to GCs of the follicle (Clayton *et al.* 1992). The production of GnRH by ovarian cell types is now well demonstrated in many vertebrate species. It has recently been shown that oocytes of the gilthead sea bream (Wong & Zohar 2004), rat and mouse (Schirman-Hildesheim *et al.* 2005) produce and release gonadotrophin together with GnRH. The available data suggest that ovarian GnRH receptors are located on oocytes or GCs (Uzbekova *et al.* 2002). A further *in vitro* study showed that the treatment with GnRH analogue enhanced, whereas the treatment with GnRH antagonist reduced the gonadotrophin release from the oocyte of the Gilthead Sea bream (Wong & Zohar 2004). Collectively, these observations support the intriguing possibility that ovarian

Figure 5 All figures are the transverse section of the ovary of the bird, *Estrilda amandava*. (A) Ovary showing GnRH I immunoreactivity mainly in the granulosa cells and the oocyte of growing follicles. (B) Late vitellogenic follicle shows GnRH I immunoreactivity in the periphery of oocyte, granulosa cells and thecal cells. (C) Preabsorbed control ovarian section showing no GnRH I immunostaining in the granulosa cells as well as in the oocyte of secondary follicle. (D) Ovary showing GnRH I-receptor immunoreactivity in the granulosa cells and oocyte of secondary follicles. (E) Early vitellogenic follicle shows GnRH I-receptor immunoreactivity in the granulosa cells and oocyte. (F) Late vitellogenic follicle showing GnRH I-receptor immunoreactivity in the granulosa cells and periphery of oocyte. (G) Ovary showing bradykinin immunoreactivity mainly in the granulosa cells and periphery of the oocyte of growing follicles. (H) Late vitellogenic follicle showing bradykinin immunoreactivity in the periphery of oocyte and granulosa cells. (I) Preabsorbed control ovarian section showing no bradykinin immunostaining in the oocyte and granulosa cells of secondary follicles. (J) Ovary shows bradykinin receptor immunoreactivity in the granulosa cells of secondary follicles. (K) Early vitellogenic follicle showing bradykinin receptor immunoreactivity in the granulosa cells and periphery of oocyte. (L) Late vitellogenic follicle enlarged to show bradykinin receptor immunoreactivity in the periphery of oocyte, granulosa cells and thecal cells.

GnRH–gonadotrophin axis may be involved in bidirectional communication between oocytes and their companion somatic cells during follicular and/or oocyte development.

The results of this study clearly demonstrate the presence of GnRH I-receptor in the ovaries of all the vertebrate species (fish, frog, reptile and bird). Among vertebrates, the presence of GnRH I-receptor has previously been demonstrated mainly in the ovaries of several species of fishes: African catfish, *Clarias gariepinus* (Habibi *et al.* 1994), sea bream, *Sparus aurata* (Nabissi *et al.* 1997), goldfish, *Carassius auratus* (Pati & Habibi 1998) and a teleost, *Fugu rubripes* (Moncaut *et al.* 2005). The presence of GnRH I-receptor in the vertebrate ovary suggests that GnRH I acts through a receptor-mediated process (Schirman-Hildesheim *et al.* 2005). Unlike the pituitary GnRH I-receptor, ovarian receptors are most likely activated by locally produced GnRH I, since this peptide is not found in detectable amounts in the peripheral circulation. Within the ovary, GnRH I-receptor immunoreactivity was mainly found in the GCs and oocytes, which are the sites of GnRH immunolocalization. This is in agreement with a study on the rat ovary, where both GnRH I and GnRH

I-receptor mRNA are demonstrated to be present in the GCs of the growing follicles, suggesting the involvement of GnRH I in follicular growth and selection (Kogo *et al.* 1999). Similarly in mammalian testis, GnRH I is produced by the Sertoli cells, whereas GnRH I-receptor is found on the Leydig cells (Bahk *et al.* 1995). These observations suggest that GnRH receptors are closely located to the site of GnRH synthesis in the ovary as well as in other extrahypothalamic loci. These findings suggest the co-evolution of GnRH I/GnRH I-receptor together as a coordinated functional unit. Since GnRH receptors are localized in multiple sites in the ovary, it can be suggested that GnRH receptor might be involved in the control of various ovarian functions. In addition to the direct effects of the GnRH-receptor in activating intracellular signalling, recent studies have suggested a cross-talk of GnRH-receptor with some growth factor receptors such as epidermal growth factor (EGF)-receptor (Shah *et al.* 2003). It is now well known that various paracrine and autocrine factors functionally interact with one another in a highly coordinated fashion (Leung *et al.* 2003). Thus, the actions of GnRH within the ovary are diverse and need further detailed investigation.

Figure 6 Slot blot for GnRH I and western blot for GnRH I-receptor. Densitometric analysis of the data showed higher ($P < 0.01$) intensity of GnRH I immunostaining in the brain of fish and amphibian as compared with reptile and bird. Intensity of GnRH I immunostaining in the ovary observed was significantly higher ($P < 0.01$) in reptile and bird when compared with fish and amphibian. Western blot analysis for GnRH I-R in the ovary of the reptile showed higher ($P < 0.05$) intensity of immunostaining when compared with the ovary of fish, amphibian, bird and the pituitary of rat. Values are significantly different with (a) fish, (b) amphibian, (c) reptile, (d) bird and (e) rat pituitary.

In the present study, GnRH I and GnRH I-receptor concentrations were compared in the ovaries of various vertebrate species (fish, frog, reptile and bird). The result showed relatively higher intensities of GnRH I and GnRH I-receptor in the ovaries of the reptile, *C. versicolor*, when compared with the other vertebrate species. Interestingly, the intensities of GnRH I-receptor in the ovary of the reptile were found significantly higher than the intensities of GnRH I-receptor in the rat pituitary lysate. This finding suggests that the significance of GnRH I in the ovary is no less than its classical role in the pituitary to stimulate the synthesis and release of gonadotrophins. The reason for the higher GnRH

I/GnRH I-receptor concentration in the ovary of the reptile is not known, it may be correlated with the higher yolk accumulation in their ovary. The GnRH I may have other important functions in the oocyte of the reptiles, which require further detailed investigation.

Despite the fact that the roles of bradykinin in the mammalian ovary are well recognized (Kihara *et al.* 2000), little is known about the presence of peptide in the ovary of non-mammalian vertebrates. The present study demonstrates for the first time the presence of bradykinin as well as BK-R using immunocytochemistry and western blotting in the ovaries of different vertebrate species (fish, frog, reptile and bird). In all the vertebrate

Figure 7 Slot blot for bradykinin and Western blot for bradykinin receptor. Densitometric analysis of the data showed higher ($P < 0.05$) intensity of immunostaining in the brain of amphibian and bird when compared with reptile. The ovary of the reptile showed higher ($P < 0.05$) intensity of immunostaining for bradykinin when compared with the ovary of fish, amphibian and bird. The bradykinin receptor showed two bands in the rat pituitary as well as in the ovary of all vertebrate species. The upper band predominates in amphibian, reptile and bird and showed higher ($P < 0.05$) intensity of immunostaining for bradykinin receptor when compared with fish, while the lower band showed comparatively higher ($P < 0.05$) intensity of immunostaining for bradykinin receptor in the fish when compared with the amphibian, reptile and bird. Values are significantly ($P < 0.05$) different with (a) fish, (b) amphibian, (c) reptile, (d) bird and (e) rat pituitary.

species studied, both bradykinin and bradykinin receptors are localized in the ovarian follicles where GnRH I is also present. This suggests the possibility that ovarian bradykinin may be regulating GnRH release in the ovary as has been the case in the rat hypothalamus (Shi *et al.* 1998). The presence of BK-R in the ovarian cells along with GnRH I immunoreactivity further supports this possibility, although more detailed studies are needed to confirm this hypothesis.

Bradykinin may induce a number of effects in the ovary. Previous studies have demonstrated that bradykinin can induce ovulation or potentiate the effect of LH in rat ovary (Yoshimura *et al.* 1988, Brännström & Hellberg 1989). Besides these, bradykinin may induce the release of prostaglandins, cause inflammatory reaction, vasodilation, etc. and may partially stimulate oocyte maturation through its effect on GnRH (Ekholm *et al.* 1981). Although in an earlier study the ovary of frog has failed to show any modulating effect of bradykinin on 17β -oestradiol and prostaglandin E_2 production, the presence of immunoreactivity in the ICs of the ovaries may suggest the involvement of this peptide in the steroidogenesis in this vertebrate ovary. Both bradykinin and BK-R are found to be localized mainly in the oocytes, GCs and TCs of the growing follicle. This suggests the physiological importance of bradykinin in the early stages of the follicular development as demonstrated for the mammalian ovary. Intrafollicular bradykinin-producing system is present in the porcine ovary (Kihara *et al.* 2000). Further, bradykinin has been noticed predominantly in the secondary follicles, which are more sensitive to the growth as well as atresia (Erickson *et al.* 1985). Bradykinin causes dilation of the blood vessels resulting in the increase in permeability of the secondary follicles, which in turn enhances the transfer of plasma substance into the surrounding extravascular spaces and also into the oocytes. It should, however, be noted that the enlargement of the follicles due to increased permeability is observed throughout follicular development and is particularly pronounced in the later stages.

In summary, the major findings of this study are the demonstration of both mRNA and peptide of mammalian GnRH I in the ovaries of fish, frog, reptile and bird. The ovaries of fish, frog, reptile and bird showed GnRH I immunoreactivity and showed more or less similar pattern of distribution in these vertebrates. GnRH was mainly localized in the oocyte of early growing follicles and GCs and theca cells of large maturing previtellogenic follicles. Mature vitellogenic follicles showed mild or no immunoreactivity. GnRH I-receptor, bradykinin and bradykinin receptor also showed immunoreactivity in the same cell types. The ovaries of reptiles showed relatively higher intensities of immunoreactivity for GnRH I and GnRH I-receptor by western blot as compared with the ovaries of fish, amphibia and bird. Maximum GnRH I/GnRH I-receptor immunoreactivities

prior to the vitellogenic phase and a transient rise in immunoreactivity were found in the previtellogenic and vitellogenic follicles, suggesting the possibility of GnRH I in the process of vitellogenesis and follicular development in the vertebrate ovary. This study further suggests the possibility of bradykinin as a regulator of ovarian GnRH I.

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