Two Molecular Forms of Gonadotropin-Releasing Hormone from the Brain of the Frog, Rana ridibunda: Purification, Characterization, and Distribution*

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ABSTRACT

The molecular forms of GnRH in amphibia have not been characterized structurally. An antiserum to mammalian GnRH that shows appreciable cross-reactivity with chicken GnRH-I ([Glu6]GnRH), chicken GnRH-II ([His6,Trp7,Tyr9]GnRH), and salmon GnRH ([Trp7, Leu8]GnRH) was used in a RIA to detect GnRH-related peptides in an extract of the brain of the European green frog, Rana ridibunda. Two peptides were purified to apparent homogeneity, and determination of their primary structures showed that they are identical to mammalian GnRH and chicken GnRH-II. Salmon GnRH and lamprey GnRH-I ([Tyr7,Leu8,Glu5,Trp7,Lys8]GnRH) were not identified in this species of frog. Immunocytochemical analysis using specific antisera has identified regions of the frog diencephalon and telencephalon in which chicken GnRH-II and mammalian GnRH are localized to different populations of neurons. This differential distribution suggests distinct physiological roles for the two forms of GnRH in amphibia. (Endocrinology 132: 2117–2123, 1993)

GnRH IS a decapetide, first isolated from pig (1) and sheep (2) hypothalamic tissue, that is present in the brains of species from all classes of vertebrates (reviewed in Ref. 3), with the possible exception of the hagfish (Agnatha) (4). Although mammalian tissues contain posttranslationally modified derivatives of GnRH (5), nucleotide sequence analysis of cloned cDNAs from humans, rats, and mice (reviewed in Ref. 6) have identified only one gene encoding prepro-GnRH, which contains a single copy of GnRH. In contrast, brain tissues from several nonmammalian species contain at least two distinct, but structurally similar, GnRH peptides (3, 7). At the present time, however, cDNA clones encoding only one GnRH precursor have been have isolated from chickens and two species of teleost fish despite the fact that a second form of GnRH exists in the species (6). Immunohistochemical and RIA studies using antisera raised against mammalian GnRH have identified GnRH-like immunoreactivity in the hypothalamic and extrahypothalamic brain of diverse species of amphibia (reviewed in Ref. 8). On the basis of chromatographic and immunochemical properties, it was suggested that peptides identical to mammalian GnRH (9, 10), chicken GnRH-II (10), and salmon GnRH (9) are present in frog brain, but structural characterization of these peptides has not been described.

A previous report (11) described the purification, by gel permeation chromatography and reverse phase HPLC, of urotensin-II from the brain of the European green frog, Rana ridibunda. In the present study, we examined side-fractions from this purification for the presence of peptides with GnRH-like immunoreactivity and isolated in pure form two such components.

Materials and Methods

Materials

Synthetic peptides were supplied by Peninsula Laboratories (Belmont, CA). HPLC columns were obtained from Separations Group (Hesperia, CA). Pyroglyutamyl aminopeptidase from calf liver (excision grade) was purchased from Calbiochem (San Diego, CA).

Tissue extraction

Adult frogs (Rana ridibunda) were obtained from a commercial source (Coutard, St. Hilaire de Riez, France). Whole brain was removed from 1200 specimens, and the tissues were immediately frozen on dry ice. The extraction of frog brain (9.4 g wet weight) by boiling in 0.5 M acetic acid has been described in detail previously (11). Peptide material was isolated by adsorption onto Sep-Pak C-18 cartridges (Waters Associates, Milford, MA), and bound material was recovered by elution with 70% (vol/vol) acetonitrile-water and lyophilized.

RIA procedure

GnRH-like immunoreactivity was measured, as previously described (12), using antiserum R1245 with mammalian GnRH as standard and [125i]iodotrotyl mammalian GnRH as tracer. The antiserum was raised against mammalian GnRH and shows 65%, 19.5%, 4.2%, and less than 1 × 10^{-6} % reactivity with chicken GnRH-I, salmon GnRH, chicken GnRH-II, and lamprey GnRH-I, respectively. GnRH-like immunoreactivity was also measured, as previously described (13), using antiserum 1467 raised against lamprey GnRH-I with lamprey GnRH-I as standard...
and [3H]iodosulphate-lamprey GnRH-I as tracer. The antisera shows approximately 0.02% reactivity with mammalian GnRH and chicken GnRH-II.

Purification of GnRH from frog brain

The brain extract, after partial purification on Sep-Pak cartridges, was redissolved in 1% (vol/vol) trifluoroacetic acid-water (10 ml) and chromatographed on a 2.5 x 100-cm column of Sephacryl S-100 (Pharmacia-LKB, Uppsala, Sweden) equilibrated with 1 M acetic acid at a flow rate of 120 ml/h. Fractions (10 ml) were collected, and absorbance was measured at 280 nm. Fractions previously shown to contain urostenin-II (11), with a K_\text{av} between 0.95-1.05, were pooled and purified further by reverse phase HPLC on a semipreparative C-18 column under the conditions described in Fig. 1. The fractions containing GnRH-like immunoreactivity (peaks I and II; shown by the bars in Fig. 1) were separately chromatographed on an analytical C-4 reverse phase column under the conditions described in Fig. 2. The fractions containing GnRH-like immunoreactivity were chromatographed on an analytical phenyl column, as described in Fig. 3. The frog GnRH peptides were purified to apparent homogeneity by a final chromatography on an analytic C-18 reverse phase column (Fig. 4).

Structural characterization

Amino acid compositions were determined by precolumn derivatization with phenyl isothiocyanate, using an Applied Biosystems model 420A derivatizer, followed by separation of phenylthiocarbamyl amino acids by reverse phase HPLC (14). Hydrolysis in 5.7 M hydrochloric acid (24 h at 110 C) of approximately 250 pmol peptide was carried out. The purified frog GnRH peptides (~400 pmol) were separately incubated for 12 h at 37 C with 1 ug pyroglyutamyl aminopeptidase in 0.1 M sodium phosphate buffer, pH 8.0, containing 5% (vol/vol) glycerol, 10 mM EDTA, and 5 mM dithiothreitol (total volume, 100 uL). The reaction mixtures were chromatographed on a 0.21 x 25-cm Vydac 218TP52 C-18 column equilibrated with 0.1% (vol/vol) trifluoroacetic acid-water at a flow rate of 0.3 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 28% (vol/vol) over 60 min (data not shown). The amino acid compositions of the Des-[pGlu]GnRH-II peptides were determined by automated Edman degradation in an Applied Biosystems model 471A sequencer (Foster City, CA) modified for detection of amino acid phenylisothiocyanate derivatives under gradient elution conditions. The detection limit was 0.5 pmol. Mass spectrometry was performed at the Novo/Nordisk Research Institute (Bagvaerd, Denmark) using a API III LC/MS system (Sciex, Thornhill, Ontario, Canada). The triple quadrupole instrument is fitted with a pneumatically assisted electrospray interface, and sample was introduced through a fused capillary (75-mm id) at a flow rate of 0.5 uL/min using a syringe infusion pump (Sage Instruments, Cambridge, MA). Approximately 100 pmol of each peptide was used. The instrument was calibrated with the singly charged ammonium ion adducts of poly(propylene glycol). and the accuracy of mass measurement was 0.02%.

Immunocytochemical studies

Adult male frogs were perfused transcardially with 30 ml 0.1 M PBS, pH 7.4, containing 0.025% xylocaine and with 50 ml McLean's fixative (15). The brains with attached pituitaries were dissected and postfixed in McLean's fixative for 3 h. Tissues were immersed in PBS containing 15% (wt/vol) sucrose for 12 h and then transferred to PBS containing 30% (wt/vol) sucrose. The brains were immersed in OCT Tissue Teck (Reichert-Jung, Nussloch, Germany), and frontal or sagittal serial sections at a thickness of 8 mm were cut in a cryostat at -20 C (Jung Microtome, model 2800E, Leica, Rueil Malmaison, France). Tissue slices were mounted on glass slides coated with 0.5% gelatin-5% chromium and processed by the indirect immunofluorescence technique, as previously described (16). Adjacent sections were incubated at 22 C for 12 h in a moist chamber with an antisera raised against mammalian GnRH (17) or with antisera raised against chicken GnRH-II (18). The antisera were used at a dilution of 1:200 in 0.1 M PBS containing 0.03% (vol/vol) Triton X-100 and 1% (wt/vol) human serum albumin. The sections were washed with PBS and incubated for 1 h at 22 C with fluorescein isothiocyanate-conjugated antirabbit \gamma-globulin (Nordic Immunology, Tuburg, The Netherlands) at a dilution of 1:60. The sections were rinsed in PBS and mounted with 50% (vol/vol) PBS-glycerol. Slides were examined using a Leitz Orthoplan microscope (Rockleigh, NJ) with a Vario-Orthomat photographic system (Leitz, Wetzlar, Germany). The following specificity controls were performed: 1) replacement of the primary antisera with PBS or nonimmune rabbit serum and 2) preincubation of the antisera with synthetic mammalian GnRH (10^{-6} M) or chicken GnRH-II (10^{-4} M).

Results

Purification of frog GnRH peptides

The elution profile on a semipreparative Vydac C-18 column of the extract of frog brain after partial purification on a Sephacryl S-100 column is shown in Fig. 1. Individual peaks were collected by hand and assayed for GnRH-like immunoreactivity using the antisera to mammalian GnRH and lamprey GnRH-I. As shown by the bars in Fig. 1, GnRH-like immunoreactivity, determined with antisera to mammalian GnRH, was eluted as two peaks (designated I and II) with the same retention times as mammalian GnRH and chicken GnRH-II. As the antisera shows only 4.2% reactivity with chicken GnRH-II, the apparent immunoreactivity in peak II does not indicate the true peptide content. The antisera shows moderate (19.5%) reactivity with salmon GnRH, but no GnRH-like immunoreactivity was detected at the retention time of salmon GnRH. Only two small peaks of GnRH-like immunoreactivity (<100 fmol/tube) were detected using the antisera to lamprey GnRH-I at the retention times of peak I and peak II GnRH. As the antisera shows somewhat lower reactivity with mammalian GnRH and chicken GnRH-II, the peaks probably represent the results of cross-reactivity with these peptides.

Peak I and II GnRHs were separately chromatographed on an analytical Vydac C-4 column (Fig. 2, A and B). In both cases, GnRH-like immunoreactivity was associated with a single sharp peak. After rechromatography of these fractions on an analytical Vydac phenyl column (Fig. 3, A and B), GnRH-like immunoreactivity was again associated with well defined peaks. The frog GnRH peptides were purified to apparent homogeneity on an analytical Vydac C-18 column (Fig. 4). The retention time of peak I was identical to that of mammalian GnRH, and the retention time of peak II was identical to that of chicken GnRH-II. The final yields of pure peptides, determined by amino acid composition analysis, were: peak I, 650 pmol; and peak II, 800 pmol.

Structural characterization

The amino acid composition of peak I frog GnRH [Glu, 0.8 (1); Ser, 1.4 (1); Gly, 2.3 (2); His, 0.9 (1); Arg, 1.1 (1); Pro, 1.0 (1); Tyr, 1.0 (1); Leu, 1.0 (1) residues/mol peptide] indicated its probable identity with mammalian GnRH. The values in parentheses are the corresponding numbers of residues in mammalian GnRH. The amino acid sequences of the frog GnRH peptides were determined by automated Edman degradation after removal of the N-terminal pyroglyutamyl residue with pyroglyutamyl aminopeptidase. The sequence of peak I frog GnRH was established as His(Ser)Trp(Trp)-Ser(Tyr)-Gly(Leu)-Arg-Pro-Arg-Gly(4), which is identical to Des-[pGlu]mammalian GnRH.
The values in parentheses show the yields of phenylthiohydantoin amino acids in picomoles.

The amino acid composition of peak II frog GnRH [Glx, 0.7 (1); Ser, 1.3 (1); Gly, 2.3 (2); His, 1.9 (2); Tyr, 0.9 (1) residues/mol peptide] indicated its probable identity with chicken GnRH-II. The sequence of peak II frog GnRH was established as His(87)-Trp(91)-Ser(23)-His(48)-Gly(78)-Trp(11)-Tyr(13)-Pro(5)-Gly(3), which is identical to Des-[pGlu¹] chicken GnRH-II.

The structures of the frog GnRH peptides, including the presence of an α-amidated glycine residue, were confirmed by mass spectrometry. The observed molecular mass (M₀) of peak I GnRH was 1181.5 compared with a calculated M₀ of 1181.5 for mammalian GnRH, and the observed M₀ of peak II GnRH was 1236.0 compared with a calculated M₀ of 1236.0 for chicken GnRH-II.

Distribution of GnRH-containing neurons

The distribution of GnRH-containing neurons in the frog diencephalon and telencephalon was studied by the indirect immunofluorescence techniques using antisera specific for chicken GnRH-II and mammalian GnRH. A dense accumulation of chicken GnRH-II-immunoreactive perikarya and processes was observed in the dorsal infundibular nucleus (Fig. 5A). In contrast, this nucleus was devoid of mammalian GnRH-containing elements (Fig. 5B). In the telencephalon, dense bundles of immunoreactive fibers were visualized in the nucleus medialis septi (NMS) using both antisera (Fig. 5, C and D). Scattered cell bodies with mammalian GnRH immunoreactivity were also detected in the NMS (Fig. 5D), but adjacent sections were devoid of chicken GnRH-II-immunoreactive perikarya (Fig. 5C). Globally, cell bodies and fibers with chicken GnRH-II immunoreactivity were widely distributed in the diencephalon, including the nucleus posterior centralis thalami, the preoptic nucleus, and the dorsal infundibular nucleus. A dense accumulation of chicken GnRH-II-immunoreactive fibers was also found in the neurointermediate lobe of the pituitary. In contrast, cell bodies immunoreactive for mammalian GnRH were found exclusively in the telencephalon, and very few fibers were detected in the diencephalon. As previously reported by Andersen et al. (17), no fibers containing mammalian GnRH were observed in the intermediate lobe of the pituitary. Preabsorption of the antisera with the homologous peptide resulted in complete abolition of immunostaining. However, preabsorption of the antiserum to mammalian GnRH with chicken GnRH-II and preabsorption of the antiserum to chicken GnRH-II with mammalian GnRH did not prevent immunostaining.

Discussion

The present study contributes to our knowledge of the molecular evolution of GnRH-related peptides. Previous work has led to the isolation and structural characterization of GnRH from an agnathan (sea lamprey) (19), holoeelaphan (Pacific ratfish) (20), elasmobranch (spiny dogfish) (21), and teleost (salmon (22) and catfish (23)) fish, a reptile (American alligator) (24), and a bird (chicken) (25, 26). The amino acid sequences of these peptides have been compared in a recent article together with a hypothetical schema describing their evolution from an ancestral peptide (21). We now demonstrate that an amphibian, Rana ridibunda, contains two GnRH peptides that are identical to mammalian GnRH and chicken GnRH-II. The data indicated that salmon GnRH and lamprey GnRH-I were not present in this species of frog. The absence of GnRH-like immunoreactivity measured with the antiserum raised against lamprey GnRH-I is significant in light of the observation that the human hypothalamus contained peptide material that was detected with the same antiserum (27).
Fig. 2. Purification of peak I frog GnRH (A) and peak II frog GnRH (B) on an analytical reverse phase HPLC column. The peptides were injected onto a 0.46 × 25-cm Vydac 214TP54 C-4 column equilibrated with 0.1% (vol/vol) trifluoroacetic acid-water at a flow rate of 1.5 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 28% (vol/vol) over 45 min using a linear gradient. The bars denote the peak containing GnRH-like immunoreactivity, and the arrows indicate where peak collection began and ended. ABS, Absorbance.

Existing data indicate that the molecular forms of GnRH isolated from *R. ridibunda* brain arose early in vertebrate evolution and have been strongly conserved during evolution. The presence of chicken GnRH-II has been demonstrated unambiguously by amino acid sequence analysis in cartilaginous fish [rathfish (20) and spiny dogfish (21)], and chromatographic evidence for its presence in phylogenetically ancient bony fish (reedfish, sturgeon, and alligator gar) has been provided (28). Mammalian GnRH has not been identified in cartilaginous fish, but chromatographic evidence
indicates that it is the predominant molecular form in the ancient bony fish (28). It has been suggested, therefore, that both forms were present in the phylogenetic ancestor of bony fish and tetrapods (28). Attempts to isolate the gene or cDNA encoding the precursor of chicken GnRH-II have not yet been successful, so the precise evolutionary relationship between mammalian and chicken GnRH-II peptides remains unclear.

A previous immunohistochemical study using antisera specific for mammalian GnRH and human GnRH-associated peptide (the C-terminal flanking peptide of pro-GnRH) investigated the distribution of neurons containing mammalian GnRH in the brain of *R. ridibunda* (17). We have now extended this study by comparing this distribution with the distribution of neurons containing chicken GnRH-II. Incubation of consecutive tissue sections with antisera specific for chicken GnRH-II and mammalian GnRH revealed that the peptides are contained in distinct neuronal populations that are differentially distributed in the frog brain. Neurons with chicken GnRH-II immunoreactivity were widely distributed in the hypothalamic region and sent dense bundles of fibers into the diencephalon as well as the neural and intermediate lobes of the pituitary. This distribution is consistent with the hypothesis that the chicken GnRH-II peptide is primarily involved in the regulation of neuroendocrine processes. In contrast, neuronal cell bodies and fibers displaying mammalian GnRH-like immunoreactivity were restricted to the telencephalon, and very few fibers were detected in the diencephalon. These data are consistent with the previous...
study (17) and suggest that the mammalian GnRH peptide may function as a neurotransmitter and/or neuromodulator in this species. In support of this hypothesis, intracerebroventricular injections of mammalian GnRH in amphibria produce a range of behavioral effects, particularly on sexual receptivity (reviewed in Ref. 8). The demonstration that the two molecular forms of GnRH have separate distributions in the frog brain warrants further studies to elucidate their physiological roles in amphibia.

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