GENERAL AND COMPARATIVE ENDOCRINOLOGY 58, 231–242 (1985)

Structure–Activity Relationships of Mammalian, Chicken, and Salmon Gonadotropin Releasing Hormones *in Vivo* in Goldfish

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Accepted July 19, 1984

Mammalian, chicken, and salmon gonadotropin releasing hormones (GnRHs), and anlogs of each peptide, were injected either alone or in combination with pimozide into goldfish, and the changes in serum gonadotropin (GtH) levels determined. The native peptides had similar potencies in terms of magnitude and duration of the GtH response. Analogs of LHRH that are superactive in mammals are also superactive in goldfish; although [(imB$_2$l)-D-His^6, Pro^9-NEt]-LHRH is very highly superactive in mammals it has activity similar to [D-Ala^6, Pro^9-NEt]-LHRH in goldfish. D-Ala^6 or (imB$_2$l)-D-His^6 substitutions of [Trp^7, Leu^8, Pro^9-NEt]-LHRH are not superactive in goldfish, whereas the D-Arg^6 substitution is highly superactive, indicating that there are differences in the factors that make salmon and mammalian GnRH superactive. These results also indicate that the structural modifications that determine superactivity of GnRHs in goldfish differ from what is known for mammals.

The primary structure of a chum salmon gonadotropin releasing hormone (sGnRH) has been determined to be [Trp^7, Leu^8]-luteinizing hormone-releasing hormone (LHRH) (Sherwood *et al.*, 1983). This form of sGnRH is found in many species of teleost fish (Sherwood *et al.*, 1984). The structure of one form of GnRH from chickens has been determined as [Gln^8]-LHRH (King and Millar 1982a, b, c; Miyamoto *et al.*, 1982, 1983; designated here as bGnRH).

Relatively little information is available on the biological activity of these new neuropeptides. Injection of sGnRH into coho salmon was effective in inducing ovulation (Sherwood *et al.*, 1983), and sGnRH and LHRH were found to be equipotent in stimulating gonadotropin (GtH) release from *in vitro* perfused pituitary fragments from goldfish (MacKenzie *et al.*, 1984). In a radioreceptor assay utilizing membrane preparations from rat pituitary and ovary, sGnRH had only about 5% of the affinity of LHRH (Sherwood *et al.*, 1983). bGnRH was found to have about 4% of the potency of LHRH in stimulating LH and follicle stimulating hormone release from rat pituitary cells *in vitro* (Miyamoto *et al.*, 1982, 1983) and 1% of the potency of LHRH in stimulating LH release in a sheep pituitary cell bioassay (Millar and King, 1983); however, bGnRH and LHRH are equipotent in stimulating LH release from chicken pituitary cells *in vitro* (King and Millar, 1982a; Millar and King, 1983). In a radioreceptor assay using rat pituitary membranes, bGnRH had 2.3% of the affinity of LHRH (Milton *et al.*, 1983); bGnRH and LHRH had equal affinities for chicken pituitary membrane receptors (Millar and King, 1983).

In the present study the activity *in vivo* in goldfish of sGnRH, bGnRH, LHRH, and analogs of all three peptides was studied. Dopamine has direct GtH release-inhibi-

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0016-6480/85 $1.50

tory activity in goldfish (Chang and Peter, 1983a, b; Chang *et al.*, 1984a, b) and injection of the dopamine antagonist pimozide greatly potentiates the GtH release-response to LHRH analogs (Chang and Peter, 1983b; Sokolowska *et al.*, 1984; Chang *et al.*, 1984a). Accordingly, we have tested the GtH releasing activity of the various peptides with and without prior injection of pimozide (Pim). The results of our studies provide a basis for discussion of structure–activity relationships of sGnRH and LHRH in goldfish.

MATERIALS AND METHODS

Goldfish, common or comet varieties, 20–30 g body weight, were purchased from Ozark Fisheries, Stoutland, Missouri, at various times of year. The fish were held in a 1800-liter flow-through aquarium at 12–13° on a simulated natural (Edmonton) photoperiod for about 5 weeks. At 3 to 7 days prior to an experiment, the selected fish were transferred to 96-liter aquaria at 12–13° and a 16-hr light 8-hr dark photoperiod. Experiment I was done in November using a combination of males and females that were sexually regressed or in early stages of gonadal recrudescence (gonadosomatic index, GSI = 1.9 ± 0.2, ♂; GSI = 3.3 ± 0.4♀) divided into two subsets. (Note: it was not possible to externally sex the fish, necessitating use of mixed sexes.) Experiment II was done in February using male goldfish (GSI = 3.7 ± 0.1), divided into three subsets. Experiments III and IV were done in April and May, respectively, using male goldfish (April, GSI = 4.0 ± 0.1; May, GSI = 4.2 ± 0.2).

The peptides used in the various experiments, and the identification code for each are listed in Table 1. LHRH and LHRH-A were purchased from Sigma. LHRH-B, sGnRH, GnRH-A to F, and bGnRH (Fig. 2b) were synthesized by J. Rivier and W. Vale. bGnRH (Figs. 1b and 2c) and bGnRH-A were synthesized by R. Millar. The peptides were dissolved in acidified (pH 6.0) fish physiological solution (PS; Burnstock, 1958) for intraperitoneal injection. The peptide dosages in Experiments I and II were all at 0.1 μg/g body weight; dosages in Experiments III and IV are given in the results. Injection volumes of PS in control fish, and of peptides dissolved in PS, were 5 μl/g body weight.

Pim (gift from Janssen Pharmaceuticals Ltd., Beerse, Belgium) was made up as a suspension in a vehicle (Veh) of acidified 0.7% NaCl with 0.1% sodium metabisulfite (Chang and Peter, 1983a). Control fish were injected with Veh. In Experiments I, II, and IV, Pim was injected intraperitoneally at 10 μg/g body weight, similar to Chang and Peter (1983a, b) and Sokolowska *et al.*, (1983); in Experiment III the dosage was 1 μg/g body weight. In each of the experiments injections of Veh or Pim were given at 0800 hr, and the peptides or PS injections were given 3 hr later (1100 hr). This protocol was used because in pilot studies it was found that injection of Pim and LHRH-A, with this interval, was highly effective in potentiating the GtH release-response to LHRH-A. Serial blood samples (100–150 μl) were taken, as described by Chang and Peter (1983a); the number of samples and the sampling times are given in the Results for each experiment. The fish were anesthetized (Chang and Peter, 1983a) for each injection and blood sampling; the fish were killed by spinal transection to determine GSI following the last blood sample.

Serum GtH concentrations were determined by radioimmunoassay (Peter *et al.*, 1984). Mann–Whitney *U* test (two-tailed) was used to compare GtH concentrations between groups.

RESULTS

The results of Experiment I are shown in Figs. 1a, b. Injection of LHRH and sGnRH (0.1 μg/g body weight) at 3 hr after Veh injection caused a significant increase in serum GtH levels at 1 and 3 hr postinjection compared to the Veh–PS control group (Fig. 1a). Injection of Pim–LHRH caused a significant increase in serum GtH levels at 1, 3, and 24 hr compared to Veh–PS and Pim–PS control groups, and at 3 and 24 hr compared to the Veh–LHRH group. Injection of Pim–sGnRH caused a significant increase in serum GtH levels at 1, 3, and 24 hr compared to Veh–PS controls, at 1 and 3 hr compared to Pim–PS controls, and at 1 hr compared to the Veh–sGnRH group. There were no significant differences between the Veh–LHRH and Veh–sGnRH groups, or between the Pim–LHRH and Pim–sGnRH groups. The Veh–bGnRH and Veh–bGnRH-A groups had significant increases in serum GtH levels compared to Veh–PS controls at 1 and 3 hr; at 24 hr the Veh–bGnRH-A group had significantly higher levels than the Veh–PS group. The Pim–bGnRH and Pim–bGnRH-A groups had significantly higher serum GtH at 1, 3, and 24 hr compared to the Veh–PS controls; only at 1 hr was there a significant

TABLE 1
THE PRIMARY STRUCTURE OF LHRH AND OTHER PEPTIDES USED IN THE STUDY. AN IDENTIFICATION CODE IS GIVEN FOR EACH PEPTIDE.

	1	2	3	4	5	6	7	8	9	10
LHRH	pGlu–	His–	Trp–	Ser–	Tyr–	Gly	–Leu–	Arg–	Pro–	Gly-NH_2 2AcOH
LHRH-A					–	D-Ala	–			–NEt
LHRH-B					–(imB_z1)-D-His–					–NEt
sGnRH							–Trp–	Leu–		
GnRH-A					–(imB_z1)-D-His–		Trp–	Leu		–NEt
GnRH-B								Trp		
GnRH-C							–Trp–	Leu–		–Gly-OH
GnRH-D							–Gln–	Leu–		
GnRH-E					–	D-Ala	–Trp–	Leu–		–NEt
GnRH-F					–	D-Arg	–Trp–	Leu–		–NEt
bGnRH								Gln–		
bGnRH-A					–	D-Trp	–	Gln–		

difference between these two groups and the Pim–PS control group, and only at 24 hr was there a significant difference between the Pim–bGnRH and Veh–bGnRH groups. Comparing the results in Figs. 1a and b, there were no significant differences between the Veh–PS control groups. There were no significant differences between the Pim–LHRH, –sGnRH, –bGnRH, and –bGnRH-A groups at any sample time; among the Veh–peptide groups, the serum GtH levels in the Veh–bGnRH-A group remained significantly elevated at 24 hr compared to the other 3 groups.

In Experiment II, done in February using males, there were no significant differences between the Veh–PS control groups at the same sample time (compare Figs, 2a, b, c); likewise, there were no significant differences between the Pim–PS control groups. As shown in Fig. 2a, the responses to Veh–LHRH and Veh–sGnRH were similar, although sGnRH did not cause a significant increase in serum GtH levels until 48 hr postinjection. The serum GtH levels in the Pim–LHRH and Pim–sGnRH groups were significantly greater than the levels in the Veh–LHRH and Veh–sGnRH groups, respectively, and the Pim–PS group, at several sample times. As shown in Fig. 2a, the injection of Veh–GnRH-A caused no significant changes in serum GtH levels; however, there was a significant response to Pim–GnRH-A, similar in magnitude to the responses to Pim–LHRH and Pim–sGnRH. Injection of Veh–GnRH-B caused no stimulation of serum GtH levels (Fig. 2a); injection of Pim–GnRH-B caused a significant increase compared to Veh–PS and Pim–PS controls at only 24 hr.

As shown in Fig. 2b, injection of Veh–GnRH-C did not cause a significant change in serum GtH levels; however, injection of Pim–GnRH-C caused a significant increase, at nearly all sample times, compared to the Veh–PS, Pim–PS, and Veh–GnRH-C groups. Notably, the response to Pim–GnRH-C (Fig. 2b) was similar in magnitude to the responses to Pim–LHRH and Pim–sGnRH (Fig. 2a). Injection of Veh–GnRH-D caused a significant increase in serum GtH levels, compared to Veh–PS controls, at 3 and 48 hr postinjection. Injection of Pim–GnRH-D caused a significant increase in serum GtH levels at all except for one of the sample times compared to the Veh–PS, Pim–PS, and Veh–GnRH-D groups. Notably, at the 24 hr sample, the response to Pim–GnRH-D (Fig. 2b) was some 3- to 3.5-fold greater than the responses to Pim–LHRH and Pim–sGnRH (Fig. 2a), respectively. Injection of Veh–GnRH-E (Fig. 2b) caused a significant increase in serum GtH levels at the 3 and 6

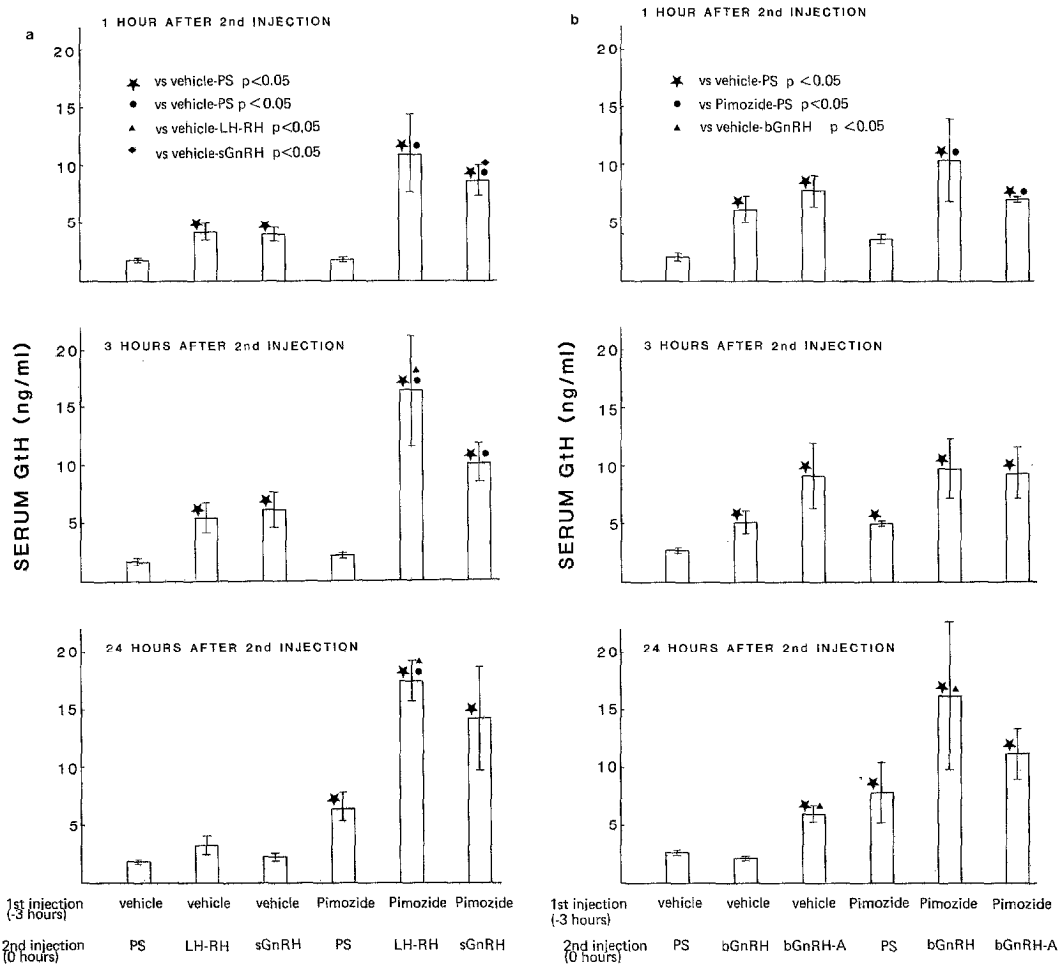


FIG. 1. (a, b) The effects of LHRH, sGnRH, bGnRH, and bGnRH-A (0.1 μg/g body weight), injected either alone or in combination with pimozide (10 μg/g body weight), on serum GtH levels in goldfish that were either sexually regressed or in early stages of gonadal recrudescense. Values are mean ± SE (N = 8–9).

hr sample times compared to Veh–PS controls; injection of Pim–GnRH-E caused a significant increase in serum GtH levels compared to the Veh–PS, Pim–PS, and Veh–GnRH-E groups at several sample times each. The magnitude of the response to Pim–GnRH-E (Fig. 2b) was similar to the responses to Pim–LHRH and Pim–sGnRH (Fig. 2a). Injection of Veh–bGnRH caused a significant increase in serum GtH levels at only the 3 hr sample time in the experiment shown in Fig. 2b, and had no significant effects in the experiment shown in Fig. 2c. Injection of Pim–bGnRH (Figs. 2b and c) caused a significant increase in serum GtH levels compared to Veh–PS, Pim–PS, and Veh–bGnRH groups at a number of sample times each; the magnitude of the response to Pim–bGnRH (Figs. 2b and c) was similar to the responses to Pim–LHRH and Pim–sGnRH (Fig. 2a). Injection of Veh–GnRH-F (Fig. 2b) caused a significant increase in serum GtH levels compared to Veh–PS controls at all sample times. Injection of Pim–GnRH-F caused a significant increase in serum GtH levels compared to the Veh–PS, Pim–PS, and Veh–GnRH-F groups at nearly all sample

times; notably, the response to Pim–GnRH-F was of great magnitude at the 24 and 48 hr sample times, varying from 6 to 18 times greater than the responses to Pim–LHRH and Pim–sGnRH.

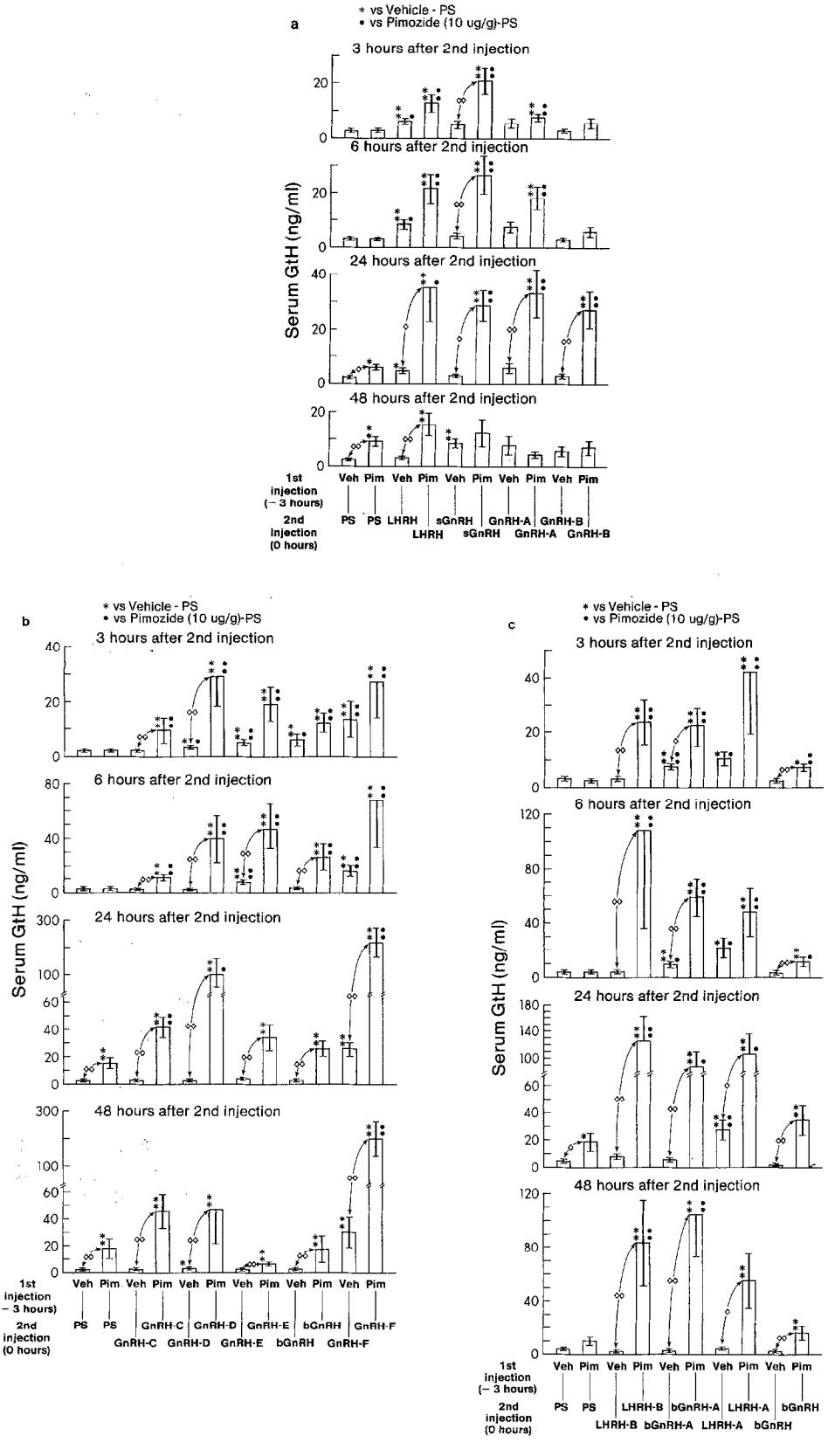
As shown in Fig. 2c, injection of Veh–LHRH-B had no significant effects on serum GtH levels. However, injection of Pim–LHRH-B caused a significant increase compared to the Veh–PS, Pim–PS, and Veh–LHRH-B groups at all sample times; notably, the response to Pim–LHRH-B was of great magnitude at the 24 hr sample time, varying from 3.5-fold greater than the response to Pim–LHRH to 4.5-fold greater than the response to Pim–sGnRH. Injection of Veh–bGnRH-A caused a significant increase in serum GtH levels compared to the Veh–PS controls at the 3 and 6 hr sample times. Injection of Pim–bGnRH-A caused a significant increase compared to the Veh–PS, Pim–PS, and Veh–bGnRH-A groups at all sample times; notably, the response was of great magnitude relative to the responses to Pim–LHRH and Pim–sGnRH (Fig. 2a) at the 24 and 48 hr sample times, varying from 2.4- to 8.0-fold greater. Injection of Veh–LHRH-A (Fig. 2c) caused a significant increase in serum GtH levels compared to Veh–PS controls at the 3, 6, and 24 hr sample times. Injection of Pim–LHRH-A caused a significant increase compared to the Veh–PS, Pim–PS, and Veh–LHRH-A groups at nearly all sample times; the response to Pim–LHRH-A was of great magnitude relative to the responses of Pim–LHRH and Pim–sGnRH (Fig. 2a) at the 24 hr sample time, varying from 3.1- to 3.8-fold greater, respectively.

In Experiment III (Fig. 3) done in April using sexually mature males early in the normal spawning season, a lower dosage of Pim was used than in the other experiments. Pim–LHRH caused a significant increase in serum GtH levels at only the 4 hr sample; Pim–sGnRH did not cause a significant stimulation in serum GtH levels at any sample time. With Pim–GnRH-F differences in the response between dosages were evident at each sample time, although only at the 48 hr sample was there a significant difference between all three doses. With Pim–LHRH-A differences in the response between dosages were evident at each sample time, but there were no sample times at which all three dosages were significantly different. At the 48 hr sample there were significant differences between the Pim–GnRH-F and Pim–LHRH-A groups at both the 0.1 and 0.01 μg/g dosages of the peptides. There is parallelism of the log dose–response curves to Pim–GnRH-F and Pim–LHRH-A at both the 24 and 48 hr sample times (figures not shown), allowing potency comparisons; GnRH-F is 7 and 17 times more potent than LHRH-A at the 24 and 48 hr sample times, respectively.

Experiment IV (Fig. 4) was done in May using males in the prime of spawning season. Injection of Veh–LHRH-A (0.1 μg/g) and Veh–GnRH-F (0.1 μg/g) both caused a significant increase in serum GtH levels compared to the VEH–PS control group, as in Experiment II. When given with Pim (10 μg/g) each dosage of LHRH–A and GnRH–F caused a significant increase in serum GtH levels of great magnitude; also, there were no significant differences between dosages of each peptide, or between the peptides, when given with Pim.

DISCUSSION

As in previous studies using female goldfish (Chang and Peter, 1983b; Chang *et al.*, 1984a; Sokolowska *et al.*, 1984, 1985) injection of Pim (10 and 1 μg/g body weight) to block the inhibitory actions of dopamine on GtH release, greatly potentiated the response to injection of LHRH-A using male goldfish (Experiments II, III, and IV). The lower dosage of Pim (1 μg/g body weight), while still effective in potentiating the actions of LHRH-A, does not cause as great a potentiation as the larger dosage (Chang


a
* vs Vehicle - PS
• vs Pimozide (10 ug/g)-PS
3 hours after 2nd injection
6 hours after 2nd injection
24 hours after 2nd injection
48 hours after 2nd injection
Serum GtH (ng/ml)
1st injection (– 3 hours)
2nd injection (0 hours)
Veh Pim Veh Pim Veh Pim Veh Pim Veh Pim
PS PS LHRH sGnRH GnRH-A GnRH-B
LHRH sGnRH GnRH-A GnRH-B
b
* vs Vehicle - PS
• vs Pimozide (10 ug/g)-PS
3 hours after 2nd injection
6 hours after 2nd injection
24 hours after 2nd injection
48 hours after 2nd injection
Serum GtH (ng/ml)
1st injection (– 3 hours)
2nd injection (0 hours)
Veh Pim Veh Pim Veh Pim Veh Pim Veh Pim Veh Pim
PS PS GnRH-C GnRH-D GnRH-E bGnRH GnRH-F
GnRH-C GnRH-D GnRH-E bGnRH GnRH-F
c
* vs Vehicle - PS
• vs Pimozide (10 ug/g)-PS
3 hours after 2nd injection
6 hours after 2nd injection
24 hours after 2nd injection
48 hours after 2nd injection
Serum GtH (ng/ml)
1st injection (– 3 hours)
2nd injection (0 hours)
Veh Pim Veh Pim Veh Pim Veh Pim Veh Pim
PS PS LHRH-B bGnRH-A LHRH-A bGnRH
LHRH-B bGnRH-A LHRH-A bGnRH

and Peter, 1984a; M. Sokolowska and R. Peter, unpublished results), probably because it only partially blocks the actions of dopamine. Although dosages of Pim greater than 10 μg/g body weight have not been tested, the data from Chang and Peter (1983b), Chang *et al.* (1984a), and Sokolowska *et al.* (1984, 1985) indicate that this dosage provides maximal inhibition of dopamine and maximal potentiation of the actions of LHRH-A.

Peter (1980) and Chang and Peter (1983b) found that a pair of injections of a low dosage of LHRH or LHRH-A was more effective in stimulating circulating levels of GtH in goldfish than a single injection of a higher dosage; a single injection caused only a very modest or no significant stimulation of the GtH levels. This indicates that self-potentiation of action of GnRH peptides can occur. Nevertheless, the response to pairs of injections of LHRH and LHRH-A at similar dosages in goldfish caused increases in serum GtH levels of similar magnitude, although the response to LHRH-A was maintained longer than for LHRH (Peter, 1980). In the present study, a single injection of LHRH or LHRH-A, without Pim, caused only modest increases of similar magnitude in serum GtH levels (Experiment II). However, when LHRH or LHRH-A were given as a single injection following Pim, the action of each was potentiated, but the response to Pim–LHRH-A was several fold greater than the response to Pim–LHRH (Experiments II and III). LHRH-A is well known as a superactive analog of LHRH in mammals (Sandow *et al.*, 1978; Vale *et al.*, 1981), and although it has been shown to be somewhat more active than LHRH in goldfish (Peter, 1980), trout (Crim *et al.*, 1981), and coho salmon (Van Der Kraak *et al.*, 1983), a major difference in the magnitude of the GtH release-response was not found in these studies. The present study indicates that, in goldfish at least, it is necessary to block the inhibitory actions of dopamine with Pim in order to evaluate the potency of different GnRH peptides.

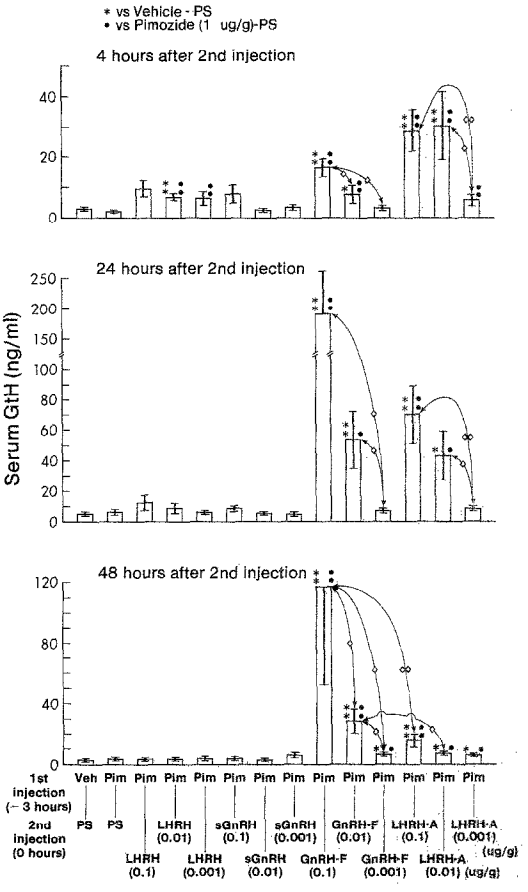


FIG. 3. The effects of different dosages LHRH, sGnRH, GnRH-F, and LHRH-A injected at 3 hr after injection of pimozide on serum GtH levels in male goldfish that had completed testicular recrudescence. Values are mean ± SE ($N = 7–9$). Significance levels: one symbol, $P < 0.05$; two symbols, $P < 0.01$.

From the results of Experiments I and II, it is evident that LHRH, sGnRH, and

FIG. 2. (a, b, c) The effects of LHRH, sGnRH, bGnRH, and analogs of each peptide (0.1 μg/g body weight), injected either alone or in combination with pimozide, on serum GtH levels in male goldfish that had completed testicular recrudescence. Values are mean ± SE ($N = 8–9$). Significance levels: one symbol, $P < 0.05$; two symbols $P < 0.01$.

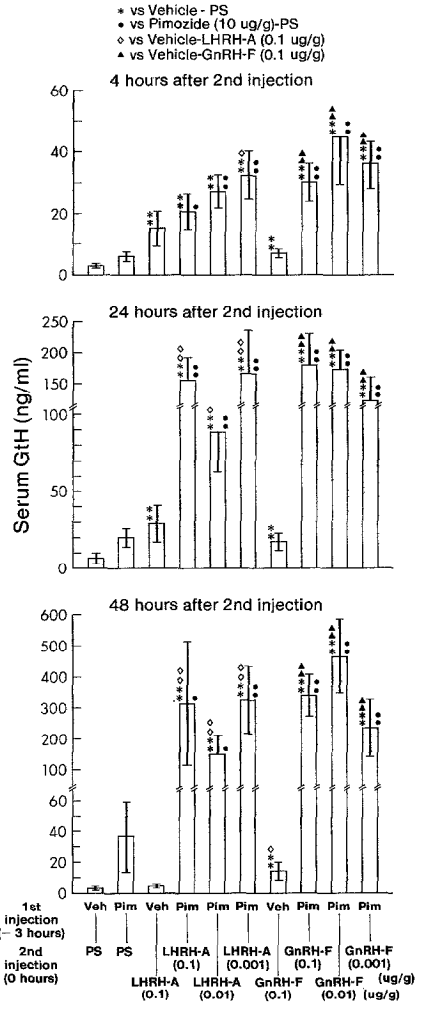


FIG. 4. The effects of LHRH-A and GnRH-F injected either alone or in combination with pimozide on serum GtH levels in male goldfish that had completed testicular recrudescence. Values are mean ± SE ($N = 8–10$). Significance levels: one symbol, $P < 0.05$; two symbols, $P < 0.01$.

bGnRH, injected 3 hr after injection of Pim, each caused an increase in serum GtH levels similar in terms of magnitude and duration. Injection of these three peptides without Pim each caused only a very modest increase in serum GtH levels, or, in some experiments, no significant effects at all. This suggests that pituitary GnRH receptors in goldfish generally do not distinguish between position 7 and 8 substituted LHRH peptides. Contrary to this, however, [Trp^8]-LHRH (GnRH-B), the reverse amino acid sequence in positions 7 and 8 of sGnRH, was relatively inactive, supporting that the authentic primary structure of sGnRH is [Trp^7, Leu^8]-LHRH, as proposed by Sherwood *et al.* (1983). Also, [Gln^7, Leu^8]-LHRH (GnRH-D), the reverse sequence of bGnRH, when given with Pim, was more active than both LHRH and sGnRH given with Pim; the magnitude of the response to Pim-GnRH-D suggests that this peptide is superactive in goldfish. On the other hand, [Gln^7, Leu^8]-LHRH may be found as a natural GnRH in goldfish; [Trp^7, Leu^8]-LHRH has been demonstrated to be present in several teleost species (Sherwood *et al.*, 1984), it is likely that [Trp^7, Leu^8]-LHRH is a natural GnRH in goldfish as dilutions of goldfish brain extracts are parallel to standards in a [Trp^7, Leu^8]-LHRH radioimmunoassay (R. Peter, C. Nahorniak, and W. Vale, unpublished results).

In mammals analog of LHRH with aromatic D-amino acids, such as imidazole benzl-D-His ((imB$_z$l)-D-His) or D-Trp, substituted at position 6, combined with Pro^9-*N*-ethylamide (Pro^9-NEt) at the C-terminus have potencies 100-fold or greater than LHRH (Sandow *et al.*, 1978; Vale *et al.*, 1981). Pro^9-NEt analogs with D-Ala^6 or D-Arg^6 substitutions have potencies about 10- to 30-fold greater than LHRH. The results of Experiment II demonstrate that [(imB$_z$l)-D-His^6, Pro^9-NEt]-LHRH (LHRH-B) and [D-Trp^6, Gln^8]-LHRH (bGnRH-A) given in combination with Pim are more active (2- to 6-fold at 24 hr sample, 5- to 8-fold at 48 hr sample) than LHRH and sGnRH, and, although not $\geq$100-fold more active as in mammals, are judged to be superactive in the goldfish. However, [D-Ala^6, Pro^9-NEt]-LHRH (LHRH-A), given in combination with Pim, has about the same activity as LHRH-B and bGnRH-A. This indicates that the presence of aromatic D-amino acid substitutions in position 6 of

LHRH does not affect the potency of LHRH analogs in goldfish as they do in mammals. This is supported by the finding in Experiment II that [(imB_zl)-D-His^6, Trp^7, Leu^8]-LHRH (GnRH-A), given in a combination with Pim, has activity similar to (Pim-)LHRH and (Pim-)sGnRH. Although the hydrophobic nature of sGnRH (Sherwood *et al.*, 1983) may affect the nature of the substitutions required to make this peptide superactive, the results with LHRH-A, LHRH-B, and bGnRH-A indicate that either their affinity for goldfish pituitary GnRH receptors and/or the metabolic degradation of GnRH peptides in the goldfish differs from what is known for mammals (Sandow *et al.*, 1978; Vale *et al.*, 1981).

In mammals the Gly^{10}-OH C-terminus configuration of LHRH has less activity than LHRH itself (Sandow *et al.*, 1978). In Experiment II [Trp^7, Leu^8, Gly^{10}-OH]-LHRH (GnRH-C) caused no changes in serum GtH levels when given alone (Veh–GnRH-C); however, when given with Pim, this peptide did cause an increase of similar magnitude as Pim–LHRH and Pim–sGnRH. This indicates that GnRH-C has about the same activity as LHRH and sGnRH, and reinforces the idea that the structure–activity relationships of GnRH peptides differ between goldfish and mammals.

[D-Arg^6]-LHRH is about 4-fold more potent than LHRH in mammalian assay systems (Sandow *et al.*, 1978). Unfortunately, we did not include this analog of LHRH in our study. As demonstrated in Experiment II, [D-Arg^6, Trp^7, Leu^8, Pro^9-NEt]-LHRH (GnRH-F), given with Pim, was superactive in stimulating GtH release in goldfish. Notably, the magnitude and duration of the increase in serum GtH levels in goldfish following Pim–GnRH-F tended to be significantly greater than for any of the other superactive peptides combined with Pim. Veh–GnRH-F and Veh–LHRH-A in Experiment II stimulated similar increases in serum GtH levels at the 3, 6, and 24 hr samples, but at the 48 hr sample the serum GtH levels of the Veh–GnRH-F group were significantly higher than in all other Veh–peptide groups. Thus, even in the absence of Pim GnRH-F is still the most active peptide of all those tested.

In Experiment III the activities of LHRH, sGnRH, GnRH-F, and LHRH-A, in combination with a low dose of Pim (1 μg/g body weight), were compared. A very small response to the two lower dosages of LHRH occurred at the time of the 4 hr sample, but not at other samples, and there was no detectable response to sGnRH. Both GnRH-F and LHRH-A caused a dose-dependent increase in serum GtH levels; dose–response comparisons indicate that GnRH-F has a greater potency than LHRH-A, confirming that GnRH-F is more superactive. A clear dose-dependent relationship between dosage of GnRH peptide and circulating GtH levels was not found in previous *in vivo* studies on goldfish (Peter, 1980; Lin *et al.*, 1983), coho salmon (Van Der Kraak *et al.*, 1983), and common Carp (Weil *et al.*, 1975), although one was reported for common carp by Breton and Weil (1973) and brown trout by Crim *et al.* (1981). Pim also potentiates the GtH release-response to LHRH-A in common carp (Billard *et al.*, 1983), and coho salmon (G. Van Der Kraak and J. Chang, personal communication). For goldfish, and perhaps also for other teleosts, it is apparently necessary to partially block the inhibitory actions of dopamine on GtH release with a low dose of Pim in order to study the dose-dependent effects of GnRH peptides.

In contrast to the results in Experiment III, in Experiment IV similar dosages of LHRH-A and GnRH-F were given in combination with a large dose of Pim (10 μg/g body weight), and no dose response was evident. Experiment III was done with males early in the normal spawning season (April) and Experiment IV was done with males during the time of normal spawning season (May). Lin *et al.* (1983) demon-

strated a marked seasonal cycle, related to testicular condition, in the response to LHRH-A in male goldfish, with the most responsive time being when testicular recrudescence had been completed (January–February) through to the mid spawning season, and the least responsive time being when the fish were sexually regressed. The greatest potentiation by Pim of the *in vivo* GtH release-response to LHRH-A in female goldfish is found in fish that have completed or nearly completed ovarian recrudescence, and the least potentiation occurs in sexually regressed males and females (Sokolowska *et al.*, 1985). In Experiment I goldfish in a sexually regressed state or in early gonadal recrudescence were used. The response to Pim (10 μg/g body weight)–bGnRH-A in Experiment I was less than what was found in Experiment II, done with male goldfish in final stages of testicular recrudescence, supporting previous observations on seasonal variations in responsiveness. Since the male goldfish in Experiments III and IV were at a stage of sexual development such that they were maximally responsive to LHRH-A, the absence of a dose-dependent response in Experiment IV must have been because the large dose of Pim used in this experiment greatly sensitized the pituitary to GnRH peptides.

It is open for speculation as to why substitutions of aromatic D-amino acids at position 6 of [Trp^7, Leu^8, Pro^9-NEt]-LHRH do not make the peptide superactive in goldfish. One possibility is that since sGnRH is already more hydrophobic than LHRH (Sherwood *et al.*, 1983), likely due to the Trp^7, substitution of yet another aromatic amino acid makes the molecule hydrophobic to such an extent that it becomes bound to lipids and is not easily available for binding with receptors. Another question is why [D-Arg^6, Trp^7, Leu^8, Pro^9-NEt]-LHRH (GnRH-F) is superactive and [D-Ala^6, Trp^7, Leu^8, Pro^9-NEt]-LHRH (GnRH-E) is not superactive in goldfish, as these position 6 substitutions of [Pro^9-NEt]-LHRH have similar superactivity in mammals (Sandow *et al.*, 1978; Vale *et al.*, 1981). Alanine is a neutral amino acid, and it will be of interest to determine whether position 6 substitution of other acidic D-amino acids confers superactivity similar to [D-Arg^6, Trp^7, Leu^8 Pro^9-NEt]-LHRH (GnRH-F) in goldfish. Perhaps position 6 substitution of an acidic D-amino acid in [Trp^7, Leu^8, Pro^9-NEt)-LHRH influences both its metabolic degradation and receptor binding affinity in goldfish.

Using perfused goldfish pituitary fragments, LHRH, sGnRH, and LHRH-A were found to be equipotent (MacKenzie *et al.*, 1984). The present results demonstrate that LHRH and sGnRH have similar activity *in vivo* in goldfish. However, LHRH-A is superactive *in vivo* in goldfish, suggesting that the decreased degradation of this peptide may be the primary factor determining its potency *in vivo*, and not its pituitary receptor binding affinity. In mammals, on the other hand, LHRH-A has increased binding affinity and a decreased degradation rate both contributing to its superactivity (Sandow *et al.*, 1978; Vale *et al.*, 1981; Loumaye *et al.*, 1982). Our results indicate that the factors that determine superactivity of specific GnRH peptides in the goldfish differ from what has been established for mammals. Additional studies are necessary to determine the basis for superactivity of GnRH peptides *in vivo* in goldfish and other teleosts.

ACKNOWLEDGMENTS

We wish to thank Dr. B. Breton for the purified carp gonadotropin used in radioimmunoassays. This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada to R.E.P., National Institutes of Health to J.E.R. and W.W.V., and Medical Research Council of South Africa to R.P.M. and J.A.K.

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