GnRH stimulates LH release directly via inositol phosphate and indirectly via cAMP in African catfish

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Rebers, Frank E. M., Peter T. Bosma, Wytske van Dijk, Henk J. T. Goos, and Rüdiger W. Schulz. GnRH stimulates LH release directly via inositol phosphate and indirectly via cAMP in African catfish. Am. J. Physiol. Regulatory Integrative Comp Physiol. 278: R1572–R1578, 2000.—In African catfish, two gonadotropin-releasing hormone (GnRH) peptides have been identified: chicken GnRH (cGnRH-II) and catfish GnRH (cfGnRH). The GnRH receptors on pituitary cells producing gonadotropic hormone signal through inositol phosphate (IP) elevation followed by increases in intracellular calcium concentration ([Ca\(^{2+}\)]). In primary pituitary cell cultures of male African catfish, both cGnRH-II and cfGnRH dose dependently elevated IP accumulation, [Ca\(^{2+}\)], and the release of the luteinizing hormone (LH)-like gonadotropin. In all cases, cGnRH-II was more potent than cfGnRH. The GnRH-stimulated LH release was not associated with elevated cAMP levels, and forskolin-induced cAMP elevation had no effect on LH release. With the use of pituitary tissue fragments, however, cAMP was elevated by cfGnRH, and forskolin was able to stimulate LH secretion. Incubating these fragments with antibodies against cfGnRH abolished the forskolin-induced cAMP elevation but did not compromise the forskolin-induced cAMP elevation. This suggests that cfGnRH-containing nerve terminals are present in pituitary tissue fragments and release cfGnRH via cAMP signaling on GnRH stimulation, whereas the GnRH receptors on gonadotrophs use IP/[Ca\(^{2+}\)] to stimulate the release of LH.

The GnRH receptors are G protein-coupled receptors that mainly signal via activation of phospholipase C, resulting in the production of inositol phosphates (IP; see Ref. 16 for review); GnRH-dependent IP formation has been shown in gonadotrophs from rat (34) and in primary cultures of goldfish gonadotrophs (35). On stimulation of IP formation has also been observed in pituitary cell lines transfected with GnRH-R cDNA constructs, such as GH3 cells stably transfected with rat GnRH-R (13). Also, in nonpituitary cells equipped with a GnRH-R, such as COS cells transfected with the human GnRH-R (24) or human embryonic kidney (HEK293) cells transfected with the catfish GnRH-R, elevation of IP levels has been observed on GnRH stimulation (36). Inositol phosphates elevate intracellular calcium concentrations ([Ca\(^{2+}\)]) by release from intracellular stores (34). Primary cultures of mammalian gonadotrophs show spontaneous [Ca\(^{2+}\)] oscillations (35). On stimulation with GnRH, the oscillation frequency increases, and at higher GnRH concentrations, a rapid peak increase is followed by a plateau of increased [Ca\(^{2+}\)] (34). In goldfish gonadotrophs, a similar pattern was observed (25). A subset of the goldfish gonadotrophs reacted with a transient rise followed by a plateau phase, whereas other gonadotrophs reacted with a series of [Ca\(^{2+}\)] increases. In contrast to the situation...
in mammals, the [Ca\textsuperscript{2+}] changes in goldfish gonadotrophs depend on influx of extracellular calcium, but the two native GnRHs cause a different response (5, 14). In calcium-depleted medium, cGnRH-II-stimulated LH release is blocked, whereas salmon GnRH-induced LH secretion is only partially inhibited (14). Similarly in tilapia hybrids (Oreochromis niloticus × O. aureus) and in African catfish, gonadotropin release was reduced when pituitaries were stimulated with GnRH in the presence of a voltage-sensitive calcium channel blocker (22, 38).

Besides the calcium-signaling pathway, activation of adenylate cyclase and production of cAMP have also been implicated in the mediation of GnRH effects. However, the direct involvement of cAMP in GnRH-R signaling in gonadotrophs is a matter of debate. In gonadotrophs from goldfish (6), rat (7), or αT3 cells (16), direct cAMP elevation on GnRH stimulation was not shown. Nevertheless, stimulation of goldfish gonadotrophs with cAMP induced the secretion of LH; however, this effect was additive to the GnRH-stimulated LH release (15). Stimulation of rat gonadotrophs with cAMP results in an elevation of the GnRH-stimulated LH secretion due to recruitment of gonadotrophs to the pool of releasing cells (4). Primary gonadotroph cultures of tilapia hybrids showed an increase in cAMP levels after stimulation with GnRH (21). In S9F cells stably transfected with the rat GnRH-R, cAMP elevation was observed with a maximum after 2 h, IP levels in this system being maximally stimulated after 30 min (9). In GH3 cells transfected with the rat GnRH-R, elevation of cAMP was observed only after 24 h (19). Also, HEK293 cells transfected with the catfish GnRH-R and a cAMP responsive reporter gene were shown to react with elevated cAMP levels after GnRH stimulation (36). These data indicate that the GnRH-R has the intrinsic capacity to activate the cAMP-generating system, whereas this capacity is not used in all cellular contexts. Moreover, cAMP may have effects in gonadotrophs that do not (directly) interfere with the GnRH signaling pathway.

Earlier studies on GnRH signal transduction in African catfish gonadotrophs indicated the involvement of cAMP (37). However, in these studies, the nonendogenous LH releasing hormone was used, and the experimental setup did not exclude juxtaglucine effects between pituitary cells or a nongonadotroph origin of cAMP. In the present study, we reexamined the generation of second messengers, including besides cAMP, also IP and [Ca\textsuperscript{2+}], on stimulation with the two endogenous GnRHs of the African catfish.

**MATERIALS AND METHODS**

**Fish.** In this study mature male African catfish, Clarias gariepinus, 8–12 mo of age were used. The fish were bred and raised in the laboratory hatchery as described before (11), except that a catfish pituitary extract instead of human chorionic gonadotropin was used to induce ovulation.

Chemicals. All chemicals and solutions used in the cell/tissue culture procedures were from Gibco (Gaithersburg, MD) or Sigma Chemical (St. Louis, MO) unless stated otherwise. Other chemicals were from Sigma or Merck (Darmstadt, Germany).

**Cell and tissue cultures.** Primary pituitary cell cultures were prepared as described before (20), and 250,000 cells were plated out into 24-well culture plates (Costar; Acton, MA) in 800 µL L-15 medium (15 mM HEPES buffered pH 7.4, 26 mM sodium bicarbonate, 1% (vol/vol) penicillin/streptomycin). After 1.5 h, when the cells were attached to the plate, 200 µL horse serum (HS) were added to a final concentration of 5% (vol/vol). The GnRH-stimulated LH release was studied as described previously (2). All cultures were performed at 25°C in 5% CO\textsubscript{2} in air at saturated humidity.

To study changes in [Ca\textsuperscript{2+}], or cAMP, gonadotrophs were enriched to ~75% by Percoll density gradient centrifugation (10). Cells were rinsed four times with PBS (10 mM with 15 mM NaCl, pH 7.4) to remove the Percoll. For c(A2+), studies, 30,000 cells in 50 µL L-15 were put on glass coverslips in six-well culture plates (Costar) and cultured at 25°C, 5% CO\textsubscript{2} for 1.5 h, after which 3 mL L-15 with 5% HS were added. For cAMP measurements, 125,000 cells were plated out in 24-well culture plates and processed as described above.

**Pituitary tissue fragment cultures.** Pituitary tissue fragment cultures were performed by cutting the pituitaries into quarters and incubating each quarter in 500 µL L-15 with 5% HS overnight at 25°C and 5% CO\textsubscript{2} in air. Thereafter, the fragments were incubated under the conditions described in cAMP measurements.

**IP measurements.** For the study of IP production, the cells were prepared and cultured as described in Cell and tissue cultures, except that dialyzed calf serum was used instead of HS. After overnight culture, 700 µL medium were removed and 0.3 µCi tritiated inositol (TRK 911, Amersham; Little Chalfont, UK) in 10 µL medium were added per well. After overnight incubation, the cells were washed three times by exchanging 700 µL medium with 700 µL L-15 without bicarbonate [L-15\textsuperscript{-}]. After the third wash, 700 µL medium were removed, 150 µL 25 mM LiCl in L-15\textsuperscript{-} were added, and the cells were incubated for another 10 min at 25°C. Next, 50 µL 10× concentrated GnRH solution in 25 mM LiCl in L-15\textsuperscript{-} were added. After 1 h at 25°C, the medium was removed and the cells were lysed by incubation for 10 min on ice in 500 µL ice-cold chloroform and methanol (2:1). The chloroform/methanol mixture was transferred to 2.5-ml glass tubes, and 500 µL distilled water and 500 µL chloroform were added. After centrifugation (5 min at 2,000 g, 4°C), 500 µL of the top phase were transferred to 10-ml conic glass tubes containing 500 µL of a 1:1 Dowex slurry (1 × 8, 100–200 mesh; Fluka, Buchs, Switzerland) in distilled water. The Dowex was washed three times with 3 mL of water, after which IP was eluted by washing twice with 500 µL formic buffer (1.2 M ammonium formate, 0.1 M formic acid) and collecting the eluate. After addition of 3 mL Ultima Pro scintillation liquid (Packard; Meriden, CT) to the eluate, the vials were counted. The results are presented as percent differences after GnRH treatment compared with the control value.

**Calcium determination.** Relative [Ca\textsuperscript{2+}] determination was performed as described before (2). In short, enriched gonadotrophs attached to glass coverslips were loaded with 10 µM fura 2-AM (Molecular Probes; Eugene, OR) and 0.02% Pluronic (Molecular Probes) for 1 h at room temperature and washed four times with PBS (10 mM, 0.8% NaCl, 1 mM CaCl\textsubscript{2}, pH 7.4). The coverslip was transferred to a Leiden tissue culture dish and superfused with PBS (1 mL/min) for at least 5 min to allow the cells to equilibrate. The cells were incubated with GnRH at the desired concentration for 2 min via the superfusion medium. Changes in [Ca\textsuperscript{2+}] were determined by dynamic video imaging using the MagiCal hardware and TARDIS software from J. oyce Loebl (Dukesway, Team Valley,
Gateshead, Tyne and Wear, UK). Because catfish gonadotrophs do not show [Ca\(^{2+}\)] oscillations (2), the response to GnRH was quantified by averaging the four ratio frames around the fluorescence ratio maximum, i.e., the peak ([Ca\(^{2+}\)]).

Results. Before stimulation, enriched gonadotrophs were rinsed three times, each time by exchanging 700 µl medium with 700 µl fresh L-15(–) after the third wash, 700 µl L-15(–), containing IBMX (final concentration of 0.3 mM) as well as GnRH or forskolin at the desired concentrations, were added. After 30 min of incubation at 25°C, the medium was removed and centrifuged for 10 min at 200 g and 4°C. The supernatant was stored at −20°C until assayed for LH content as described before (30). The cells were then lysed in 100 µl ice-cold 0.1 M HCl and stored at −20°C until assayed for cAMP.

Pituitary fragments were washed with L-15(–) and incubated for 3 h in 500 µl L-15(–) with 0.3 mM IBMX at 25°C. The medium was then collected and processed for LH quantification (basal secretion) as described before (30). The pituitary fragments were cultured for another 3 h in 00 µl L-15(–) with 0.3 mM IBMX and GnRH or forskolin in the presence or absence of 5% (vol/vol) rabbit preimmune serum (Arnel; New York) or 5% (vol/vol) rabbit antiserum raised against cGnRH (29). The medium was collected for LH quantification (stimulated secretion). From the LH levels, the stimulation factor was calculated by dividing stimulated by basal LH secretion, thus accounting for possible differences in gonadotroph number between tissue fragments. Finally, 100 µl of ice-cold 0.1 M HCl were added to the pituitary fragments, which were stored in their incubation wells under 0.1 M HCl at −20°C until assayed for cAMP content.

Determination of cAMP content was performed according to Norstedt and Fredholm (26) with some adaptations. The cell lysate was thawed and 250 µl neutralization solution [NS; in mM: 85 Tris, 214 NaCl, 8.6 EDTA (pH 7.4 at 4°C), 40 NaOH, and 50 HEPES] was added. Pituitary fragments were transferred to Eppendorf vials, after which the wells were washed with 250 µl NS that were added to the same vials. The fragments were homogenized using a pestle, centrifuged for 10 min at 200 g and 4°C, and the supernatant collected. An aliquot of the sample (cell lysate or pituitary fragment homogenate) or cAMP standard (15–8,000 fmol), 25,000 counts/min tritiated cAMP (TRK 498, Amersham), and 200 µl protein kinase A (16 µg/ml) were incubated at 4°C for 3 h. Then, 300 µl dextran-coated charcoal [1% Norit A (wt/vol), 0.1% dextran T-70 (wt/vol) in 30 mM sodium bisphosphate/60 mM disodium phosphate, pH 7.0 with 0.05% sodium azide, 0.9% NaCl, and 0.1% gelatin (all wt/vol)] were added, incubated for 5 min, and centrifuged at 5,400 g for 5 min at 4°C. The supernatant (500 µl) was counted.

Statistics. In all graphs, the values are given as means ± SE. Experiments were repeated at least three times, and representative data are presented. Multiple groups were compared by one-way ANOVA followed by Fisher’s protected least-significant difference test. For comparing two groups unpaired, double-sided Student’s t-tests were used. Both tests were calculated with StatView 4.5 for Windows (Abacus concepts, Berkeley, CA). For the nonlinear fits of concentration curves, sigmoidal dose-response curves and the EC\textsubscript{50} were calculated using GraphPad Prism 2.01 (GraphPad Software, San Diego, CA). Differences were considered statistically significant when P < 0.05.

Results

IP, calcium, and LH release. Stimulation of a primary pituitary cell culture for 1 h with cGnRH-II resulted in a dose-dependent increase in IP production (Fig. 1A). Similarly, cGnRH induced dose-dependent increases in IP production, which reached a slightly higher maximum level than found after incubation with cGnRH-II. Curve fitting resulted in an EC\textsubscript{50} of 0.82 nM for cGnRH-II and 1.74 µM for cGnRH.

Primary cultures of enriched gonadotrophs were incubated for 2 min with different concentrations of cGnRH-II and cGnRH to induce changes in [Ca\(^{2+}\)] (Fig. 1B). Fitting analysis of these data resulted in EC\textsubscript{50} of 0.34 and 89 nM for cGnRH-II and cGnRH, respectively. No difference in the plateau maximum between the two GnRHs was observed. We also tested the requirement of the presence of 1 mM extracellular calcium on 1 µM cGnRH-II- and 10 µM cGnRH-induced changes of [Ca\(^{2+}\)]. There was no significant influence of the lack of extracellular calcium on the response to both GnRHs (bars in Fig. 1B), and both GnRHs significantly elevated [Ca\(^{2+}\)] to the same extent as in the presence of 1 mM extracellular calcium.

Both cGnRH-II and cGnRH induced dose-dependent increases in the amount of LH secreted over a 30-min incubation period (Fig. 1C). Both GnRHs reached the same maximal plateau of secretion. Fitting analysis of these data resulted in EC\textsubscript{50} of 0.8 and 262 nM for cGnRH-II and cGnRH, respectively.

Gonadotropin release and cAMP–cell cultures versus tissue fragments. Stimulation of enriched gonadotrophs for 30 min with 1 µM forskolin greatly increased the intracellular level of cAMP, which, under control conditions or after GnRH stimulation, did not surpass the assay detection limit (15.6 fmol cAMP; Fig. 2A). As in previous experiments, stimulation of enriched gonadotroph cells with 0.1 µM cGnRH-II or 10 µM cGnRH resulted in a stimulation of LH release, whereas incubation with 1 µM forskolin did not significantly elevate LH secretion (Fig. 2C).

Incubation of pituitary tissue fragments, however, with 1 µM forskolin for 3 h (in the presence of preimmune serum), resulted in an increased LH release into the medium (Fig. 2D). As expected, also 10 µM cGnRH and 10 nM cGnRH-II (data not shown) elevated the LH secretion from the pituitary tissue fragments. Forskolin induced a large increase in the cAMP content of the pituitary tissue fragments (Fig. 2B). Unexpectedly, cGnRH (Fig. 2B) and cGnRH-II (data not shown) also increased the cAMP level, although to a lower extent than forskolin. Replacing the preimmune serum by an antiserum raised against cGnRH abolished the release of LH in response to forskolin (Fig. 2D). Preimmune serum had no influence on basal LH release, whereas antiserum against cGnRH attenuated basal LH secretion (Fig. 2D).

Discussion

The present study shows that both cGnRH and cGnRH-II stimulate secretion of LH from a primary pituitary cell culture in a dose-dependent manner and that elevations of IP levels and [Ca\(^{2+}\)] participate in GnRH signal transduction. Both GnRHs share a simple dose-response relationship in regards to LH release,
cGnRH-II being 325-fold more potent than cfGnRH; this difference may be attributed to the difference in affinity of these two endogenous ligands to the GnRH-R (29, 30).

In the catfish gonadotrophs, both cfGnRH and cGnRH-II give dose-dependent increases in the IP accumulation. This is in line with previous reports on responses to GnRH by gonadotrophs from goldfish (5) and rat (34). Similarly in the gonadotroph-derived cell line αT3 (1), the lactotroph-derived cell line GH3 expressing the rat GnRH-R (13), or COS-1 cells transfected with the human GnRH-R (24), elevation of IP was observed after GnRH stimulation. The present study indicates that in the African catfish also, both IP and 

**Fig. 1. Dose-response relationships after stimulation of African catfish primary cells in culture with chicken gonadotropin-releasing hormone (cGnRH)-II (○) or catfish gonadotropin-releasing hormone (cfGnRH; ●) with respect to inositol phosphate (IP) accumulation (A) or intracellular Ca²⁺ concentration ([Ca²⁺]) changes (B) and luteinizing hormone (LH) secretion (C). Bars in B represent fura 2 ratio in response to 1 µM cGnRH-II or 10 µM cfGnRH, respectively, in absence of extracellular calcium. All graphs represent means ± SE, n = 3–10 replicate experiments in A, n = 6–7 in B, and n = 6 (replicates in representative experiment) in C. *Significantly different plateau for cfGnRH compared with cGnRH-II (P < 0.05).**
tide (PACAP) may be more important in regulating cAMP levels in gonadotrophs (23, 28). Moreover, the increases of cAMP after GnRH stimulation are relatively slow (19) and follow the faster reaction of IP and \([\text{Ca}^{2+}]_i\), which correspond better to the time scale of LH release.

As regards the role of cAMP in GnRH-stimulated LH secretion, strikingly different results were obtained in the present study with catfish pituitary fragments versus primary cell cultures. In tissue fragments, but not in primary cell cultures, cAMP is elevated after the addition of GnRH, and the addition of forskolin stimulated LH release. This is in contrast to the situation in tilapia in which the results did not depend on using pituitary fragments or cell cultures (21), as GnRH always elevated cAMP levels. The results from the experiments with catfish pituitary cell cultures suggested that cAMP has no direct effect on LH secretion in catfish. To reconcile this with the observations made with catfish pituitary tissue fragments, we sought evidence for a cAMP-mediated but indirect effect on LH secretion. In this context, it is important to note that in teleost fish, the GnRH neurons directly contact pituitary gonadotrophs via their axons, whereas a portal blood vessel system connecting the median eminence and the adenohypophysis is missing (40). The pituitary fragments, as used in the present study, thus contain cfGnRH-containing nerve fibers. Indeed, we have shown that 95% of all cfGnRH present in the brain and pituitary of adult catfish is found in the pituitary (12). We hypothesize that the nerve terminals present in the catfish pituitary fragments (40) may release their stored cfGnRH on elevation of the cAMP level via a cAMP-involving pathway. Within such a model, a primary release of cfGnRH in response to electrical stimulation would result in the binding of cfGnRH to its own nerve terminals. This would elevate cAMP levels in these terminals leading to a further release of cfGnRH; such a mechanism may enable a cfGnRH release burst. Catfish GnRH will also bind to GnRH-R on gonadotrophs to stimulate LH secretion via the IP/\([\text{Ca}^{2+}]_i\)-dependent pathway.

Fig. 2. cAMP levels in gonadotroph cells (A) or pituitary tissue fragments (B) and LH release from gonadotroph cells (C) or pituitary tissue fragments (D). pi, Incubated with 5% preimmune serum; im, incubated with 5% anti-cfGnRH antiserum. All graphs show means ± SE from a representative experiment with n = 4 (A, C) or n = 11-18 (B, D). nd, Not detectable; bars sharing same letter do not differ significantly (ANOVA, followed by Fisher’s protected least-significant difference test, \(\alpha = 0.05\)).
GnRH, which, however, should not modulate the forskolin-induced elevation of cAMP levels. Moreover, cfGnRH should elevate cAMP levels in the tissue fragments (i.e., GnRH-R-equipped, cfGnRH-containing nerve terminals). Indeed, incubating pituitary tissue fragments with forskolin in the presence of antibodies against cfGnRH abolished the effect of forskolin on LH secretion but not on cAMP accumulation. Moreover, cfGnRH elevated cAMP levels in the tissue fragments. This small but significant response may reflect the contribution of the cfGnRH-containing nerve terminals to the total cAMP response evoked by forskolin, which is probably composed of contributions by several cell types in the pituitary tissue fragment. The fact that immunoneutralization of extracellular cfGnRH is able to dissociate the two effects forskolin has on tissue fragments (increased cAMP and increased LH secretion) is evidence to support the existence of a cAMP-mediated release of an LH release-inducing factor from pituitary tissue fragments of adult male African catfish exists. Because primary cultures of the pituitary gonadotrophs do not show this phenomenon, we attribute the forskolin effect to a cAMP-induced release of cfGnRH from the nerve terminals.

We cannot exclude that GnRH elevated cAMP in the pituitary fragments in cells other than the gonadotrophs. Previous work has shown, however, that catfish gonadotrophs are the only cells in a primary pituitary cell culture showing GnRH binding activity (3). We also cannot exclude that forskolin induced the secretion of a synergistic factor acting on LH release, for instance PACAP. Such a mechanism, if present, is probably of minor relevance, because antisera against cfGnRH completely abolished the forskolin effect on LH release in tissue-fragment experiments. Finally, it is interesting to note that incubation with cfGnRH antisem increased basal LH secretion, indicating that when working with tissue fragments, LH secretion in the absence of exogenous GnRH comprises the real, non-GnRH-stimulated basal LH secretion plus the LH secretion induced by the endogenous release of cfGnRH.

In summary, in the gonadotrophs in the African catfish pituitary, both cfGnRH and cGnRH-II regulate LH release by directly influencing the gonadotroph cells via IP3/Ca2+ signaling. Moreover, we propose that both GnRHs have the potential to stimulate cfGnRH secretion in the vicinity of the gonadotrophs through a cAMP-dependent mechanisms in the cfGnRH-containing, GnRH-R-expressing nerve terminals.

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