Involvement of cyclic adenosine monophosphate in the stimulation of gonadotropin secretion from the pituitary of the teleost fish, tilapia

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Summary

The present study examines the involvement of cAMP in the transduction of the short-term effect of gonadotropin-releasing hormone (GnRH) on gonadotropin release in the teleost fish, tilapia. A 5 min pulse of dibutyryl cyclic AMP (dbcAMP; 0.03–3 mM) or forskolin (0.1–10 μM) resulted in dose-dependent surges in tilapia gonadotropin (tgGTH) secretion from the perfused pituitary. The initial increase in tgGTH in response to dbcAMP (3 mM) occurred within 6 min. The concentration of cAMP in the effluent medium increased about 20-fold after a pulse of [D-Ala⁶,Pro⁹-NEt]-luteinizing hormone-releasing hormone (LHRH) (GnRHa; 100 nM). To rule out the possibility that the observed effects were due to stimulation by endogenous GnRH release from intact nerve terminals present in the fragments, further experiments were performed in primary cultures of dispersed pituitary cells. Exposure (30 min) of the cells to forskolin (0.01–1.0 μM) resulted in a dose-dependent increase in tgGTH release similar to that achieved by GnRHa (1 pM to 10 nM). Also 8-bromo cAMP (0.01–1.0 mM) evoked a dose-related increase in tgGTH release. A 3-fold increase in the release occurred in the presence of isobutylmethylxanthine (IBMX) (0.2 mM), similar to that obtained by GnRHa (1.0 nM) in the absence of IBMX. However, when combined, the increase in taGTH release was 16-fold. Moreover, exposure of the cultured cells to GnRHa (0.1 or 10 nM, 60 min) resulted in a dose-related elevation of intracellular cAMP levels and taGTH release. These results are consistent with the hypothesis that cAMP is involved as a mediator in the transduction of GnRH stimulation of gonadotropin release in tilapia, and operates in parallel or is interconnected with the system of Ca²⁺ influx, protein kinase C (PKC) activation and arachidonic acid, as previously described in this and other fish.

Introduction

The secretion of gonadotropin (GTH) from the pituitary of teleost fish is under a dual hypothalamic control. It is stimulated by two forms of gonadotropin-releasing hormone: salmon GnRH ([Trp⁷,Leu⁸]-GnRH = sGnRH) and chicken GnRH II ([His⁵,Trp⁷,Tyr⁸]-GnRH = cGnRH-II; Chang et al., 1990a) and is inhibited by hypothalamic dopamine (reviewed by Peter et al., 1986). A dual hypothalamic regulation of GTH secretion was reported also in tilapia (Gissis et al., 1991).

The cascade of intracellular events elicited by GnRH in mammalian gonadotrophs involves the
influx of Ca\(^{2+}\), its binding to calmodulin, hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) to form inositol trisphosphate (IP\(_3\)) and diacylglycerol (DAG), and activation of protein kinase C (PKC; reviewed by Conn et al., 1987; Naor, 1990). The increase in intracellular Ca\(^{2+}\) is essential for the activation of phospholipase A\(_2\) and for the formation of arachidonic acid which, together with its lipooxygenase metabolic products, may cause the release of stored LH (Naor and Catt, 1981; Naor et al., 1985; Kiesel et al., 1987; Chang et al., 1988; Naor, 1990, 1991). Some reports in mammals also implicate adenosine 3',5'-cyclic monophosphate (cAMP) as a possible intracellular mediator of GnRH action, but this association is restricted to long-term effects of GnRH and not to early phases of gonadotropin release (Cronin et al., 1984; Bourne and Baldwin, 1987a, b). Nevertheless, cAMP was found to stimulate LH release in permeabilized sheep pituitary cells within minutes, and cellular cAMP was elevated in response to GnRH (Macrae et al., 1990).

Previous studies in our laboratory have shown that Ca\(^{2+}\)-ionophore and an activator of PKC can mimic the effect of GnRH by stimulating the secretion of GTH from tilapia pituitary fragments in the presence of extracellular Ca\(^{2+}\) (Levavi-Zermonsky and Yaron, 1987; Levavi-Sivan and Yaron, 1989). The association of Ca\(^{2+}\) influx with the stimulation of gonadotropin secretion was reported also in the murrel, *Channa punctatus* (Jamaluddin et al., 1989), the African catfish, *Clarias gariepinus* (Van Asselt et al., 1989b), the carp (Mikolajczyk et al., 1990) and the goldfish (Chang et al., 1990b). In the goldfish and tilapia also arachidonic acid or its metabolites are implicated in the transduction of GnRH effect (Chang et al., 1989; Yaron and Levavi-Sivan, 1990).

The inhibition by dopamine of gonadotropin secretion in fish is reported to be relayed by receptors resembling the mammalian D\(_2\) type (Chang et al., 1984, 1990c; De Leeuw et al., 1988). These D\(_2\) type receptors have been defined biochemically by their ability to inhibit adenylate cyclase activity (Niznik, 1987). It is hypothesized, therefore, that dopamine inhibition of GTH release in fish is exerted by inhibition of adenylate cyclase activity. Such a route could be in operation if cyclic AMP is actually involved in the mediation of GnRH effect on the gonadotrophs. This work was designed to examine the possibility that GnRH effect on GTH secretion in tilapia involves the adenylate cyclase-cAMP system as an intracellular mediator operating in addition to that described above.

### Materials and methods

#### Fish

Female tilapia hybrids (*Oreochromis niloticus* × *O. aureus*), 150–200 g body weight, were collected at various times of the year from the fish farm of Kibbutz HaMaapil. The fish were kept in tanks at 18°C and an 8 h light/16 h dark photoperiod. Under such an environmental regimen gonadal recrudescence does not occur spontaneously.

#### Perifusion system

For each perifusion experiment, consisting of up to ten parallel channels, pituitaries were excised from three fish for each channel and cut into fragments of about 0.5 mm\(^3\). The fragments were transferred, together with preswollen Biogel P-2 (Bio-Rad, Richmond, CA, USA), into the perifusion chambers (0.5 ml in volume) and rinsed first for 18–20 h at 27 ± 1°C with the medium (Eagle's basal medium containing L-glutamine, 4 mM NaHCO\(_3\), 0.5 g/1 bovine serum albumin (BSA), 100 IU/ml penicillin, 0.1 mg/ml streptomycin, 0.25 μg/ml amphotericin B, 5 mM Hepes, buffered to pH 7.4). This procedure of extended rinsing is required to reach a low and stable baseline of taGTH secretion (Levavi-Zermonsky and Yaron, 1988).

GnRH\(_a\) ([d-Ala\(^6\),Pro\(^2\)-NEt]-LHRH) and db-cAMP (N\(^6\),O\(^2\)-O-dibutyryl adenosine 3'-5'-cyclic monophosphate) were dissolved directly in the perifusion medium. Forskolin was dissolved first in ethanol before further dilution in the medium, immediately prior to use. The final concentration of ethanol did not exceed 0.1%, which does not affect taGTH secretion. All these chemicals and agonists were purchased from Sigma (St. Louis, MO, USA). The osmolality of the medium was 300 mOsm. Medium samples were collected for taGTH determination every 15 min unless other-
wise stated. Other details of the perifusion system were as previously described (Levavi-Sivan and Yaron, 1989). The void volume of the system was equivalent to 10 min.

**Pituitary cell primary culture**

Pituitaries were collected aseptically from 35–40 female tilapia into the culture medium (Medium 199 containing NaHCO\(_3\) (9 mM), penicillin (100 IU/ml), streptomycin (0.1 mg/ml), nystatin (1.25 IU/ml; Beth Haemek, Israel)) to which 0.3% BSA was added and buffered to pH 7.4 with 10 mM Hepes. The glands were cut by a razor and trypsinized for 60 min at room temperature in 5 ml of trypsin-EDTA solution (0.25% trypsin and 0.02% EDTA in phosphate buffer saline containing 1% glucose (Beth Haemek, Israel)). The process was facilitated by constant shaking and frequent aspiration through a narrow pipette. The trypsinization was terminated by the addition of 1 ml of fetal calf serum. After counting and determination of viability by trypan blue exclusion, the cells were plated (2.5 or 4 × 10^5 cells/well) on a 24 multi-well plate (Cel-Cult, Sterilin, Hounslow, UK) in 1 ml of the same medium to which fetal calf serum (10%, v/v) was added. The cells were cultured for 4 days at 28°C under an atmosphere of 95% O\(_2\) and 5% CO\(_2\). The cells were then challenged for 30 or 60 min with the following agonists or drugs, dissolved in the culture medium with no serum, but containing BSA (0.1%): GnRHa (0.001–10.0 nM); 8-bromoadenosine-3',5'-cyclic monophosphate (8-Br-cAMP; 0.01–1.0 mM). Forskolin (0.01–1.0 μM), and 3-isobutyl-1-methylxanthine (IBMX; 0.2 mM) were dissolved first in ethanol and further diluted in the culture medium. The medium was collected after 30 or 60 min for taGTH determination.

**Determination of gonadotropin**

The gonadotropin of tilapia (taGTH), possibly equivalent to the salmonid GTH II (Suzuki et al., 1988a, b) was determined in the medium by a specific radioimmunoassay (RIA) according to Bogomolnaya et al. (1989), except that the second antibody employed was donkey anti-rabbit IgG bound to a magnetizable compound (Amerlex-M, The Radiochemical Centre, Amersham, UK). The bound and unbound fractions were separated on magnetic plates. The sensitivity of the assay was 0.5 ng/tube; the intra- and interassay coefficients of variation were 7.3% and 14.0%, respectively.

**Determination of cAMP**

IBMX was added to the effluent medium (3.25 ml/fraction) in a final concentration of 0.2 mM and the whole fraction was loaded on AMPREP minicolumns (Amersham International, UK). The nucleotide was eluted with 3 ml of HCl (0.1 M), the eluate was lyophilized and the dry sample was redissolved in the assay buffer (0.05 M acetate buffer, pH 5.8). cAMP concentration was determined by the Amersham cAMP [\(^{125}\)I] assay system following acetylation.

Intracellular cAMP was determined in cell culture exposed for 60 min to GnRHa or forskolin in the presence of IBMX (0.2 mM). Cells were scraped and extracted with 0.1 M HCl which was neutralized with 100 mM Tris/NaOH. The medium was boiled for 5 min. cAMP was determined in the extract and in the medium using the RIA kit as above.

**Statistical evaluation**

In some perifusion experiments, the total amount of taGTH released in response to the agonist was calculated by the summation of hormone content in 15 min fractions of the effluent medium. The difference among means in the same perifusion experiment was analyzed by one-way ANOVA followed by a simultaneous comparison among means (least significant difference).

In the time-course study, the basal secretion rate of taGTH was calculated as the average secretion rate in each channel during the final 3 h of rinsing. The data are presented as the ratio between the secretion rate after manipulation and the basal secretion rate in the respective channel.

The results of taGTH determinations in the culture experiment were analyzed by one-way ANOVA followed by a simultaneous comparison among means as above or by Student’s t-test.

**Results**

The effects of cAMP agonists on taGTH release were examined in perifusion experiments. A
5 min pulse of graded doses of dbcAMP (0.03, 0.3 and 3 mM) resulted in dose-dependent surges in taGTH secretion which lasted for 60 or 90 min, dependent on the dose. The total amount of taGTH released during the first 60 min in response to each concentration of the nucleotide is given in Table 1.

Addition of 0.1, 1 or 10 µM forskolin in a 5 min pulse evoked dose-dependent surges in taGTH release within less than 15 min and lasted for about 45 min. The total amount of taGTH released during the first 45 min in response to each concentration of the agonist is given in Table 1.

A time-course study, in which the effluent medium was sampled every 2 min, showed that the surge in taGTH evoked by a 5 min pulse of dbcAMP (3 mM) was evident after 6 min and reached a peak 4 min later. In the same experiment the response to GnRHa (10 nM) occurred 4 min earlier (Fig. 1).

A 5 min pulse of GnRHa (100 nM) was followed by a sharp peak of cAMP in the effluent medium within the first 15 min. At this point there was hardly any increase in taGTH secretion rate. In the next sample, cAMP concentration declined while taGTH reached a prominent peak (Fig. 2).

A 30 min exposure of dispersed pituitary cells to forskolin (0.01, 0.1 or 1 µM) resulted in a dose-dependent increase in taGTH output, which was similar to that evoked by GnRHa (0.001, 0.1 and 10 nM; Fig. 3). In another experiment, a dose-dependent increase in taGTH release was evoked by 8-Br-cAMP (0.01, 0.1 and 1.0 mM; Fig. 4A). The 3-fold increase in taGTH release in response to 30 min exposure to GnRHa (1.0 nM) was similar to that elicited by 0.2 mM IBMX. However, the release of the gonadotropin in response to GnRHa in the presence of IBMX was 16-fold the basal level (Fig. 4B).

60 min exposure of dispersed pituitary cells to forskolin (0.1, 1.0 or 10 µM) resulted in a dose-

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**TABLE 1**

THE EFFECT OF dbcAMP AND FORSKOLIN ON GONADOTROPIN RELEASE FROM PERIFUSED TILAPIA PITUITARY

Tilapia gonadotropin (taGTH) secretion from perifused fragments of three pituitaries (in each channel) in response to a 5 min pulse of either dbcAMP or forskolin. Before the exposure to the agonists, the fragments were perifused for 19 h at 27±1°C with the medium. This representative experiment consists of three parallel channels for each agonist concentration. The effluent medium was collected every 15 min for taGTH determination. Data are presented as mean ± SEM of the amount of taGTH released during the response to dbcAMP (60 min) or forskolin (45 min). The difference among means was analyzed by one-way ANOVA followed by a simultaneous comparison among means. Means designated by the same letter do not differ significantly from each other (p > 0.05).

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Concentration</th>
<th>taGTH release (µg/peak; mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dbcAMP</td>
<td>0</td>
<td>53.8 ± 5.5 a</td>
</tr>
<tr>
<td></td>
<td>0.03 mM</td>
<td>48.7 ± 9.8 a</td>
</tr>
<tr>
<td></td>
<td>0.30 mM</td>
<td>94.0 ± 17.8 b</td>
</tr>
<tr>
<td></td>
<td>3.00 mM</td>
<td>117.4 ± 3.9 b</td>
</tr>
<tr>
<td>Forskolin</td>
<td>0</td>
<td>57.7 ± 1.7 a</td>
</tr>
<tr>
<td></td>
<td>0.1 µM</td>
<td>53.6 ± 3.2 a</td>
</tr>
<tr>
<td></td>
<td>1.0 µM</td>
<td>102.4 ± 8.2 b</td>
</tr>
<tr>
<td></td>
<td>10.0 µM</td>
<td>231.9 ± 26.6 c</td>
</tr>
</tbody>
</table>

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Fig. 1. Time course study of gonadotropin secretion from perifused fragments of tilapia pituitary without any agonist (rhomboids), in response to a 5 min pulse (arrows) of 3 mM dbcAMP (circles), or 10 nM [α-Ala²,Pro⁵-NEt]-LHRH (GnRHa; squares). The details of the perifusion technique were as in Table 1. The effluent medium was sampled every 2 min during the experiment. Data in this experiment are presented as mean ± SEM (n = 3) of the ratio between the secretion rate after stimulation and the basal secretion rate (the mean secretion rate during the last 3 h of rinsing in the respective channel).
Fig. 2. Cyclic AMP (circles and left ordinate) and tαGTH (squares and right ordinate) concentrations in the effluent medium of perfused tilapia pituitary fragments in response to a 5 min pulse of GnRHα (100 nM; arrow). The fragments were perfused with the medium for 20 h before the introduction of the pulse and the effluent medium was collected at 15 min intervals. Other details of the perfusion technique were as in Table 1 and the presentation of the data as in Fig. 1.

Fig. 3. Gonadotropin secretion from a primary culture of dispersed pituitary cells of tilapia exposed for 30 min to (A) graded doses of 8-bromoadenosine-3',5'-cyclic monophosphate (8-β-cAMP); (B) 3-isobutyl-1-methylxanthine; 0.2 mM, IB) or GnRHα (1.0 nM; Gn) alone or in combination. Other details are as in Fig. 3.

Fig. 4. Gonadotropin release from a primary culture of dispersed pituitary cells exposed for 60 min to (A) graded doses of forskolin. Other details are as in Fig. 3 except that cells were plated at a density of $4 \times 10^5$. 

dependent increase in taGTH release and the maximal effective dose was 1.0 μM. In the same experiment, the intracellular concentration of cAMP increased proportionally to the forskolin dose (Fig. 5).

60 min exposure of dispersed pituitary cells to GnRHa (0.1 and 10 nM) resulted in a significant increase in taGTH release (Fig. 6); however, no change in the intracellular content of taGTH was noted (1.99 ± 0.24, 2.19 ± 0.21 and 1.77 ± 0.14 μg/4 × 10⁵ cells for control, 0.1 and 10 nM GnRHa, respectively). The content of cAMP in the same cells increased in proportion to the GnRHa dose (Fig. 6), but the concentration of the nucleotide in the medium was unaltered (11.38 ± 0.48, 12.20 ± 1.16 and 13.14 ± 0.97 pmol/well for control, 0.1 and 10 nM GnRHa, respectively).

Discussion

It is generally accepted that cAMP is not involved in the stimulatory effect of GnRH on LH release from the rat pituitary. This conclusion is inferred from studies on pituitary cell culture where neither the administration of cAMP or dbcAMP, nor the elevation of intracellular cAMP levels (by forskolin, cholera toxin, pertussis toxin or phosphodiesterase inhibitors) stimulated LH release, or augmented the response to GnRH (Naor et al., 1975 and reviewed by Conn et al., 1981, 1987). In the frog too, forskolin had only a slight stimulatory effect on either LH or FSH release from perifused pituitaries. However, the exclusion of Ca²⁺ from the medium eliminated even these marginal effects (Porter and Licht, 1986).

Nevertheless, by employing sodium flufenamate, which inhibits GnRH-stimulated increase in cellular cAMP, Bourne and Baldwin (1987a, b) reached a different conclusion. They claim that the effect of the nucleotide is manifest by stimulating de novo synthesis of proteins, either the gonadotropins themselves or proteins that are integrally related to the secretion of these hormones (Bourne and Baldwin, 1987a). This conclusion corroborates earlier findings that the effects of agents which activate adenylate cyclase in primary culture of rat adenohypophyseal cells are revealed after a time-lag of more than 4 h (Cronin et al., 1984). The time-course perifusion experiment in the present study shows that the effect of the nucleotide, which occurs within 6 min, differs in its kinetics from that in the rat or the frog. It reflects cAMP participation in gonadotropin release and is not limited to the late phase of hormone synthesis.

It would appear that the involvement of cAMP in GTH release, as shown in tilapia, is not exceptional; in a brief review, Davidson et al. (1991) stated that the lack of responsiveness to cAMP may be peculiar to the rat, since in intact pituitary cells of chicken, sheep and humans, forskolin induces LH release within a few minutes.

It is possible that cAMP takes part in the transduction of GnRH signal by increasing Ca²⁺ influx through voltage-dependent channels as demonstrated in other pituitary cell systems such as the AtT-20 or the GH₃ cell lines (Luini et al., 1985; Armstrong and Eckert, 1987). Nevertheless, a study on permeabilized sheep anterior pituitary cells shows that under intracellular concentration of Ca²⁺ clamped at resting level, cAMP stimu-
lated exocytosis within 5 min, and cAMP concentration in intact cells was elevated by GnRH (Macrae et al., 1990).

Upon the exposure of the perfused fragments to GnRHa, the concentrations of cAMP increased dramatically in the effluent medium (Fig. 2). Such an increase is known to reflect intracellular changes in the nucleotide (Dufau et al., 1978; Adams et al., 1979). In the same experiment the peak of tauGTH release lagged behind, as would be expected in the case of mediation by a second messenger.

The possible involvement of cAMP in GTH release in fish was briefly reported by Yu et al. (1985) showing that GTH release from perfused carp pituitaries can be enhanced by 1 mM of dbcAMP. In the catfish Clarias gariepinus too, forskolin was reported to stimulate GTH secretion and to enhance the buserelin-induced GTH release (Van Asselt et al., 1989a).

There is a possibility that the effects described above only reflect the effects of cAMP agonists on the release of endogenous GnRH from the nerve terminals present in the pituitary fragments. Indeed, exposure of goldfish pituitary fragments to forskolin (100 μM) or prostaglandin E₂ resulted in a significant increase in the release of endogenous GnRH from nerve terminals embedded in the fragments (Yu et al., 1991). The experiments conducted here with dispersed pituitary cells, in which the contact with nerve terminals is disrupted, were designed to examine this possibility.

Exposure of dispersed pituitary cells to either 8-Br-cAMP or forskolin stimulated the release of taGTH (Figs. 3 and 4A). The release increased by 300% in the presence of IBMX, similar to that elicited by 1 nM GnRHa. However, the synergism between these two agents resulted in a 16-fold increase in taGTH release (Fig. 4B). Furthermore, a 60 min exposure of the culture to GnRHa or forskolin resulted in elevated intracellular cAMP levels concomitantly with enhanced release of GTH (Figs. 5 and 6). Taken together, these results would indicate the involvement of cAMP in the transduction of GnRH signal.

GnRH forms such as sGnRH and cGnRH-II stimulate both GTH and GH release in the goldfish (Marchant et al., 1989). Therefore, the possibility that the somatotrophs present among the cultured cells contributed part of the rise in the measured cAMP cannot be excluded.

Previous studies in tilapia have already indicated that the stimulatory effect of GnRH is dependent on Ca²⁺ influx and is associated with diacylglycerol and activation of protein kinase C (Levavi-Zermonsky and Yaron, 1987; Levavi-Sivan and Yaron, 1989). It may involve phospholipase C, phospholipase A₂ and arachidonic acid as components of a second messenger system similar to that known in higher vertebrates (Yaron and Levavi-Sivan, 1990). The results presented here indicate that GnRH effect on the immediate release of GTH in the fish is also mediated by the adenylate cyclase-cAMP as a parallel or possibly an interconnected second messenger system.

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