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Original article

Calcium ions as a mediator in GnRH action on gonadotropin release in the common carp (*Cyprinus carpio* L)

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Summary — Collagenase-dispersed carp pituitary cells in a perifusion system were used to study the role of calcium ions in the mechanism of GnRH action on the release of maturational gonadotropin (GtH) in fish. The specific calcium chelator EGTA and the calcium antagonist manganese (Mn^{2+}) caused a 40% inhibition in the basal GtH release and completely blocked GnRH-stimulated GtH release. Short-term application of graded doses of calcium ionophore A23187 caused a dose-dependent increase in GtH secretion. A23187 failed to stimulate GtH secretion in the presence of EGTA. Depolarization of the membrane by K^+ caused a strong stimulation of GtH release similar to the action of GnRH. Stimulatory action of K^+ was inhibited by EGTA. These data suggest a role for extracellular calcium as an intracellular mediator in GnRH-stimulated, as well as in basal, GtH release in carp. The stimulation of GtH release by K^+ also indicates that voltage-dependent processes could be involved in this phenomenon.

GnRH / GtH / calcium / carp

Résumé — Rôle des ions calcium dans la médiation de l'action du GnRH sur la stimulation de la sécrétion de gonadotropine chez la carpe commune (*Cyprinus carpio* L). *Le rôle du calcium dans le mécanisme d'action du GnRH sur la stimulation de la sécrétion de gonadotropine maturante (GtH) a été étudié dans un système de périfusion de cellules dispersées d'hypophyses de carpe. L'EGTA, chélateur spécifique des ions calcium, et l'ion manganèse (Mn^{2+}), un antagoniste du calcium, diminuent de 40% la sécrétion basale de GtH et inhibent complètement l'action du GnRH. L'ionophore du calcium, A23187 induit une stimulation de la sécrétion de GtH dont l'intensité dépend de la dose, cette action est inhibée en présence d'EGTA. La dépolarisation des membranes par le potassium (K^+) induit une très importante stimulation de la sécrétion de GtH, comparable à celle engendrée par le GnRH. Ces résultats suggèrent que le calcium extracellulaire serait un médiateur intracellulaire dans la régulation des sécrétions tonique (sous l'action du GnRH) et basale de GtH. La stimulation de la sécrétion de GtH par K^+ indique également que des processus dépendant du voltage pourraient être mis en jeu dans ces phénomènes.*

GnRH / GtH / calcium / carpe

* Correspondence and reprints

INTRODUCTION

Gonadotropin-releasing hormone (GnRH) and its analogues stimulate the *in vivo* gonadotropin (GtH) release in teleost fish such as goldfish (Peter *et al*, 1985), brook trout (Crim and Cluet, 1974), common carp (Breton and Weil, 1973, Sokolowska, 1982), coho salmon (Van Der Kraak *et al*, 1983) and many other species. *In vitro* studies have also shown that GnRH is able to release GtH from perifused pituitary fragments and dispersed pituitary cells of goldfish (MacKenzie *et al*, 1984), and from cultured dispersed pituitary cells of carp (Ribeiro *et al*, 1983) and rainbow trout (Weil *et al*, 1986).

Numerous studies on the mechanism of GnRH action have been carried out in mammals (Hopkins and Walker, 1978; Adams *et al*, 1979; Benoist *et al*, 1981; Conn *et al*, 1981; Borges *et al*, 1983), and it is now well established that Ca^{2+}, which is an important signal transducer in numerous tissues and cell types (Rasmussen and Barret, 1984), plays the role of second messenger in GnRH action on LH release (Conn *et al*, 1987).

To our knowledge there are only 2 papers concerning the mechanism of GnRH action in fish: Levavi-Sivan and Yaron (1989) using perifused tilapia pituitary fragments and Jamaluddin *et al* (1989) working on murrel pituitary cells in static culture proposed a role for Ca^{2+} in the action of GnRH on GtH release in fish.

The aim of the present study was to find out if Ca^{2+} in the common carp (cyprinidae) acts as a second messenger in the action of GnRH on GtH release. To do this, we applied a perfusion of dispersed pituitary cells which permitted us to monitor the changes in GtH secretion in a much more dynamic manner than in static cell culture. In contrast with a perfusion of pituitary fragments, our system allowed us to detemine the level and the site of action of the drugs.

MATERIALS AND METHODS

Animals

Sexually mature male and female carp (mean body weight 5.3 kg) obtained from the Heyman Fish Farm were kept in a natural pond and then transported to the laboratory. They were placed in a thermoregulated recirculating water system and acclimated at 18 °C for at least 10 d before being used in the experiments.

Cell preparation

For each experiment 2 pituitaries (male + female) were used; the cells were prepared according to the technique previously described for trout (Weil *et al*, 1986) and adapted for carp. Pituitary glands were excised after decapitation and quickly placed in a sterile ice-cold medium (MEM-Eagle, GIBCO) buffered with 15 mmol/l Hepes (P–L Biochemicals Inc) and 9 mmol/l sodium bicarbonate. The medium had an osmotic pressure of 245 mOsm/kg and a pH of 7.7, the characteristic values of common carp blood plasma. The glands were rinsed several times with the medium and chopped into small pieces with surgical blades. The tissue fragments were transferred for dispersion to a siliconized glass-stoppered flask with 30 ml of the medium containing 0.1% (wt/vol) collagenase (Boehringer Mannheim) and 1% BSA (RIA grade, SIGMA). The mixture was incubated at 18 °C for 6 h. Dissociation was then completed mechanically by aspirating the fragments 4–5 times into a 10 ml syringe using a metal needle (0.6 x 10 cm). The cells were harvested by centrifugation at 200 g for 10 min at 18 °C and washed twice with the pre-incubation perifusion medium which contained 2% of a serum substitute (Ultroser-G: IBF), 100 U/ml of penicillin and 10 µg/ml of streptomycin (GIBCO). After final centrifugation the cells were resuspended in 350 µl of the medium and randomly distributed into 4 or 5 tubes containing 1 ml of the medium and 0.3 g of preswollen Bio-Gel P-2 (BIORAD). The cell suspen-

sion was then introduced into the perifusion columns ($10^6 \pm 1.5 \times 10^5$ live cells/column).

Perifusion system

The perifusion system has been previously described in detail by Gonnet *et al* (1988). The perifusion chambers (volume 1 ml) consisted of siliconized glass tubes, 0.9 cm in diameter, delimited by teflon plungers. Column temperature was maintained at 18 °C by thermoregulated water circulating in the jackets surrounding the columns. They were perifused with the medium which was constantly aerated with a mixture of O_2 (96%) and CO_2 (4%). The fractions were collected with a fraction collector. A maximum of 5 columns were perifused simultaneously.

Perifusion protocol

A mixture of the gel and cells was placed into each column and covered with 0.1 g of preswollen gel. The cells were perifused at a low flow rate (5 ml/h) with the pre-incubation – perifusion medium for 16 h. The medium was then replaced with medium containing no ultroser and antibiotics. In the experiment where K^+ was used, perifusion was performed with a mineral medium prepared according to Jalabert *et al* (1973). For 1 h the flow rate was gradually increased to 15 ml/h. After an additional h of perifusion at this rate, fractions were collected every 7.5 min before and between pulses of drugs or chemicals, and every 2.5 min during and 15 min after their administration. The duration of drug administration varied from 10 to 50 min. Details concerning each perifusion are given in the figure legends.

Tested drugs and chemicals

Synthetic salmon GnRH (sGnRH) synthesized by Breton *et al* (1984), Ethyleneglycol-bis-(-aminoethyl ether)*N, N, N', N'*-tetraacetic acid (EGTA; Sigma), and $MnCl_2$ were dissolved directly in the perifusion medium just prior to use. Calcium ionophore A23187 (Sigma) was diluted from 1 mmol/l stock solution in dimethyl sulphoxide (DMSO) and was shielded from light throughout use. The amount of DMSO never exceeded 0.04% (v/v). The same amount of DMSO was present in the perifusion medium passing through the control column and had no measurable effect on GtH release. In the experiment in which the effect of K^+ depolarization was tested, the amount of NaCl in the test medium was reduced accordingly so that the total concentrations of KCl and NaCl remained constant.

GtH determination and statistics

GtH levels were determined using a specific radioimmunoassay developed by Breton *et al* (1971). Profiles of GtH levels from both experimental and control columns are presented as a mean percentage ± SEM of the basal GtH secretion level (bl). Basal level of GtH release was estimated as the mean of the 4 points (fractions) which directly preceded drug application. The basal GtH levels of all the columns considered in this work varied from 19.35 to 40.13 ng/ml. During the application of secretagogues, GtH levels in control and in experimental columns were compared using Student's *t*-test for variation analysis.

RESULTS

Effect of different doses of synthetic sGnRH on GtH release (fig 1)

Three 15-min pulses of sGnRH at concentrations of 10^{-8}, 10^{-7} and 10^{-6} mol/l significantly increased the basal GtH secretion level by 80, 177 and 65%, respectively. The cells began to respond to sGnRH within 2.5 min after the beginning of the sGnRH pulse. Maximal GtH levels were reached within 5–7.5 min, but GtH release began to decline while the peptide was still present in the perifusion chamber. Basal GtH levels recovered soon after sGnRH withdrawal. In the following experiments, sGnRH was used at a concentration of 10^-

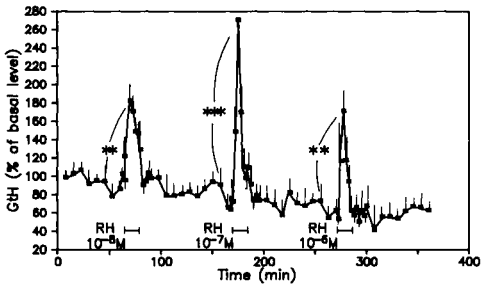

Fig 1. Effect of graded doses of sGnRH on GtH release. Ten columns (data from 3 separate experiments) received three 15-min pulses of sGnRH in concentrations 10^{-8}, 10^{-7} and 10^{-6} mol/l, as indicated. Mean basal level (bl) = 34 ± 9 ng/ml. Basal GtH level was calculated as a mean of 6 points (fractions) which directly preceded first drug application. Profiles of GtH levels are presented as a mean percentage ± SEM of the basal GtH secretion level. Significant differences in GtH secretion are indicated ($P < 0.05$ – *; $P < 0.01$ – **; $P < 0.0001$ – ***).

$^{-7}$ mol/l as the most potent stimulator of GtH release in our perifusion system.

Effect of EGTA on basal and sGnRH-stimulated GtH release (fig 2)

In the control column three 15 min pulses of sGnRH (10^{-7} mol/l) caused a significant ($P < 0.01$) increase in GtH secretion (70–90% bl). In the presence of 0.5 mmol/l EGTA, sGnRH evoked an increase in GtH secretion which was twice that of the control. At a higher concentration (5 mmol/l) EGTA caused a significant ($P < 0.05$), decrease (40% bl) of the basal GtH secretion and completely blocked sGnRH action ($P < 0.05$ and $P < 0.01$ respectively). A dramatic surge of GtH was observed 5 min after the removal of the EGTA from the perifusion medium.

Effect of duration of EGTA application on basal and sGnRH-stimulated GtH release (fig 3)

In the control column, three 15 min applications of sGnRH (10^{-7} mol/l) caused a significant ($P < 0.01$) increase in GtH secretion similar to that observed in the earlier experiment. In the experimental columns EGTA was applied (5 mmol/l) before sGnRH infusion. The first pulse of sGnRH was given after 9 min presence of EGTA in the perifusion chamber, the second after 20 min and the final one after 60 min of EGTA administration. In all cases EGTA caused a 40% decrease in basal GtH secretion ($P < 0.05$) and completely blocked the effect of sGnRH ($P < 0.01$). A dramatic surge of GtH was observed 2.5–5 min after EGTA withdrawal. The longer EGTA was present in the perifusion chamber, the stronger was the stimulation of GtH re-

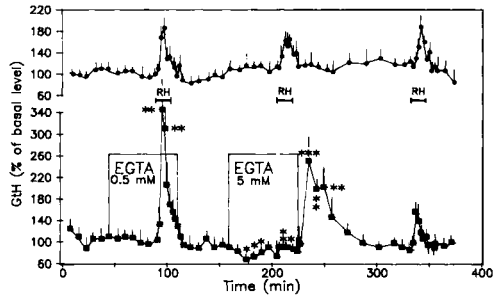

Fig 2. Effect of graded doses of EGTA on basal and sGnRH–stimulated GtH release. Control columns (●) ($n = 4$) received three 15-min pulses of sGnRH (RH; 10–7 mol/l). Mean bl = 21 ± 5 ng/ml. Experimental columns (■) ($n = 6$) were exposed to sGnRH as above but in the presence of EGTA as indicated. EGTA was present in the perifusion chamber 45 min before sGnRH application. Mean bl of experimental columns was 28 ± 4 ng/ml. Significant differences in GtH secretion between control and experimental columns during stimulation period are indicated (fig 1). Data from 2 separate experiments.

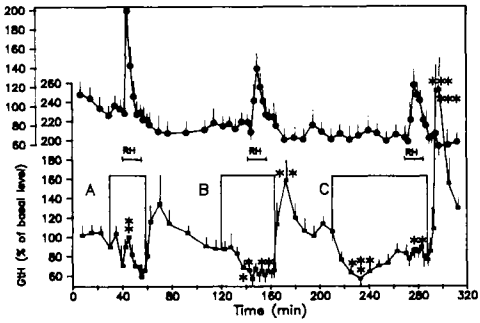

Fig 3. Effect of duration of exposure to EGTA on basal and GnRH – stimulated GtH release. Control colomns (●) (n = 4) received three 15-min pulses of sGnRH (RH; 10^{-7} mol/l). In experimental columns (■) (n = 6) sGnRH was applied in the presence of EGTA in concentration of 5 mmol/l. EGTA was administered 9 min before pulse of sGnRH (A), 20 min (B) and 60 min (C) before application of sGnRH, as indicated. Mean bl of control and experimental columns was 32 ± 10 and 29 ± 10 ng/ml respectively. Data from 2 separate experiments. For other details see figure 1.

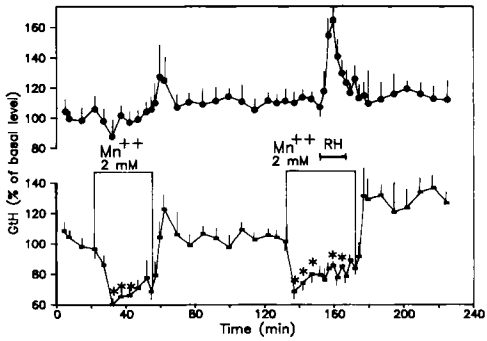

Fig 4. Effect of Mn^{2+} on basal and sGnRH – stimulated GtH release. Control columns (●) (n = 4) received a 15-min pulse of sGnRH (RH) at a dose of 10^{-7} mol/l. In experimental columns (■) (n = 6) $MnCl_2$ was applied in a concentration of 2 mmol/l for 35 and 40 min. During the second application of $MnCl_2$ a pulse of sGnRH was administered, as indicated. Mean bl of control and experimental columns was 39 ± 8 and 30 ± 8 ng/ml respectively. Data from 2 separate experiments. See figure 1 for details.

lease after EGTA withdrawal: 140, 160 and 249% of the baseline, respectively.

Effect of Mn^{2+}, a Ca^{2+} antagonist, on basal and sGnRH-stimulated GtH secretion (fig 4)

Repeated administration of $MnCl_2$ (2 mmol/l of Mn^{2+}) for 35 min (first pulse) and 40 min (second pulse) caused a 40% decrease in basal GtH secretion ($P < 0.05$). The action of 10^{-7} mol/l sGnRH (pulse given during the second application of Mn^{2+}) was completely inhibited. To compensate for a difference of osmotic pressure, the control columns received a pulse of NaCl (2 mmol/l of Na^+) at the same time as the experimental columns received $MnCl_2$. This had no significant effect on basal and sGnRH-stimulated GtH release.

Effect of calcium ionophore A23187 on GtH secretion when used alone or in combination with EGTA (fig 5)

Three 10 min pulses of A23187 at concentrations of 1, 5 and 10 µmol/l caused an increase of 0, 110 and 350% respectively, in the basal GtH secretion level. Similar pulses of A23187 given in the presence of EGTA (5 mmol/l) were strongly inhibited ($P < 0.001$) in comparison with the control columns. As in the previous experiments with EGTA, a dramatic surge of GtH was observed 5 min after the removal of EGTA.

Effect of elevated K^+ on GtH release (fig 6)

A 15 min pulse of KCl (60 mM of K^+) caused a significant ($P < 0.001$) increase

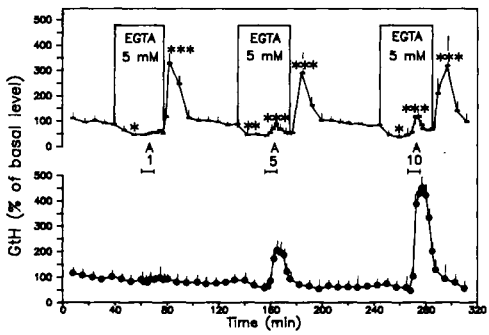

Fig 5. Effect of A23187 and EGTA on GtH release. Control columns (●) (n = 6) received three 10-min pulses of A23187 (A1, A5, A10) in concentrations of 1, 5 and 10 μM, respectively. Experimental columns (■) (n = 6) received similar pulses of A23187 in the presence of EGTA at a dose of 5 mmol/l, as indicated. EGTA was present in the perifusion chamber 20 min before A23187 application. Mean bl of control and experimental columns was 24 ± 6 and 31 ± 11 ng/ml respectively. Data from 3 separate experiments. See figure 1 for other details.

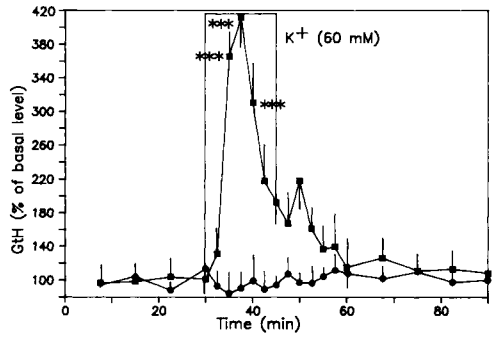

Fig 6. Effect of elevated K^+ on GtH release. Experimental columns (■) (n = 6) received a 15-min pulse of KCl in a concentration of 60 mmol/l. Six other columns (●) served as controls. Mean bl was 20 ± 6 ng/ml for control columns and 19 ± 3 ng/ml for experimental columns. Data from 3 separate experiments. See figure 1 for other details.

(315%) in GtH secretion. Maximal GtH level was reached within 7.5 min but GtH release started to decline while K^+ was still present in the perifusion chamber. Basal GtH release level was recovered 10 min after K^+ withdrawal.

Effect of EGTA on K^+-stimulated GtH release (fig 7)

A 12.5-min pulse of KCl (60 mmol/l of K^+) caused a 180% increase in GtH secretion in the control columns. In the experimental columns 5 mmol/l EGTA caused a significant ($P < 0.05$) decrease in GtH release and strongly inhibited K^+ action ($P < 0.01$). A surge of GtH occurred 5 min after EGTA withdrawal as observed in earlier experiments (figs 2, 3 and 5).

DISCUSSION

In our perifusion system the cells started to respond to sGnRH 2.5–5 min after its administration, with maximal GtH release occurring within the next 2.5 min. GtH levels started to decrease in the presence of sGnRH reaching the basal level by the end of the pulse. A concentration of 10^{-7} mol/l sGnRH was shown to be the most effective in stimulating GtH release (fig 1). A higher concentration of this peptide (10^{-6} mol/l) was less potent, indicating a possible rapid desensitization of the carp pituitary cells to high doses of sGnRH. These results as well as other results from our laboratory show that GtH is probably not released in carp in a biphasic manner as in mammals (Bremner and Paulsen, 1974; Blake, 1978). Similar results have been reported for the goldfish (Chang *et al*, 1984; MacKenzie *et al*, 1984). The results presented in figures 2 and 3 (3 pulses of

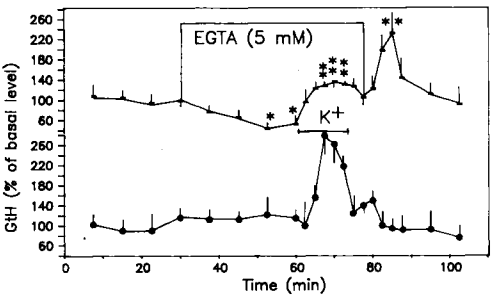

Fig 7. Effect of EGTA on K^+–stimulated GtH release. Control columns (●) (n = 4) received a 12.5-min pulse of KCl (60 mmol/l K^+). Experimental columns (■) (n = 5) received similar pulse of K^+ but in the presence of 5 mmol/l EGTA. EGTA was present in the perifusion chamber 30 min before K^+ application, as indicated. Mean bl of control and experimental columns was 14 ± 3 and 17 ± 2 ng/ml respectively. See figure 1 for other details.

sGnRH in the control columns) show that the cells could respond to repeated administration of this peptide in the same manner throughout the experiment.

To demonstrate that Ca^{2+} played a role in the action of GnRH, we used a specific Ca^{2+} chelator EGTA to chelate Ca^{2+} from the perifusion medium and also an inorganic Ca^{2+} antagonist, Mn^{2+}, antagonistic to calcium in processes independent of the voltage (Rubin, 1982). Both EGTA and Mn^{2+} completely inhibited sGnRH-stimulated GtH release and reduced basal GtH secretion by 40%. This showed that not only GnRH stimulated GtH release, but also that basal GtH secretion was Ca^{2+} dependent. In the first experiment with EGTA (fig 2), we demonstrated the total inhibition of sGnRH action on GtH release caused by EGTA when present in the perifusion chamber for 45 min before and during stimulation with sGnRH. It has been shown that such an exposure in mammals could provoke a depletion of the Ca^{2+} from the intracellular stores (Limor *et al,* 1987). In our experiments, to reveal the role of the duration of EGTA presence in its effect on GnRH action, sGnRH was applied during administration of EGTA starting 9, 20 and 60 min before addition of sGnRH. We have shown that even the shortest presence of EGTA (9 min) was able to totally inhibit sGnRH action on GtH release, indicating an extracellular origin of Ca^{2+} in the action of sGnRH. Each time a dose of 5 mmol/l of EGTA was used, a dramatic surge of GtH occurred 5 min after the removal of the drug. This could be provoked by increased Ca^{2+} permeability of the cells caused by EGTA, causing an influx of Ca^{2+} when the medium was changed. Such a situation was not observed when 2 mmol/l Ca^{2+} was added to the normal medium (data not shown). Increased Ca^{2+} permeability caused by EGTA could also be the reason why in exp 2 (fig 2) a low dose of EGTA potentiated the action of sGnRH. A dose of 0.5 mmol/l of EGTA is considered as insufficient to chelate all the Ca^{2+} from the medium (± 1.8 mmol/l of Ca^{2+}), so sGnRH-provoked Ca^{2+} influx was potentiated when the cells were exposed to EGTA. In the second experiment with EGTA (fig 3) we have also shown that the longer EGTA is present, the greater the increase in GtH secretion occurred when a Ca^{2+} containing medium is introduced. This probably means that the longer the EGTA was present, the greater was the increase in Ca^{2+} permeability, so when Ca^{2+} was introduced, there was a greater influx of Ca^{2+} and an increase in GtH secretion.

A23187 is a potent stimulating factor of LH release in mammals, mimicking the action of GnRH (Conn *et al,* 1979, 1980; Harris *et al,* 1985). In carp, A23187 induced GtH release in a dose-dependent manner (fig 5). To determine whether this stimulatory action was caused by calcium ions

transported by A23187, this drug was also administered together with EGTA. EGTA decreased the basal GtH secretion, as observed in the earlier experiments (figs 2–3) and strongly inhibited the action of A23187. A significant GtH release was observed 5 min after the removal of the EGTA.

Intracellular Ca^{2+} may rise due to the entry of extracellular Ca^{2+} *via* voltage–dependent channels (Reuter, 1983; Ovichinnikov, 1985). To reveal the possible involvement of this type of channel, the membrane can be depolarized by raising extracellular K^+. In carp, raised K^+ caused a strong stimulation of GtH release (315% bl) (fig 6). The profile of GtH release under K^+ stimulation was very similar to that obtained with sGnRH. GtH levels began to decline when K^+ was still present in the perifusion chamber, recovering the basal release level soon after K^+ withdrawal. K^+ stimulation was inhibited by EGTA (fig 7), indicating the Ca^{2+} dependence of this process. It can be supposed that the action of the depolarizing pulse of K^+ resembles the action of GnRH and indicates the possible participation of voltage–dependent Ca^{2+} channels in the mechanism of GnRH action on GtH release in carp. Our findings are in general agreement with those obtained in mammals as well as in fish. In murrel, Jamaluddin *et al* (1989) found that GnRH–stimulated GtH release requires the influx of extracellular Ca^{2+} through voltage–dependent channels. However, there are some differences between our results and those obtained by other authors. Such phenomena as an inhibition of basal GtH release by EGTA and Mn^{2+} and the strong GtH release, provoked by replacing the medium containing EGTA with normal medium, have never been observed in experiments conducted in mammals (Hopkins and Walker, 1978; Stern and Conn, 1981; Gorospe and Conn, 1987) and in fish (Jamaluddin *et al*, 1989). These differences could be a result of the different experimental system used, *ie* static culture *versus* perifusion and pituitary fragments *versus* dispersed cells.

Marian and Conn (1979) proposed 3 conditions necessary for a role of Ca^{2+} as the second messenger in GnRH action:

– the removal of calcium from its site of action would block the effect of GnRH on LH release;

– LH release would occur as a consequence of inserting Ca^{2+} into the gonadotrophs in the absence of GnRH;

– GnRH would stimulate the movement of Ca^{2+} into specific sites; this is a requisite of LH release. Our results fulfil the first 2 conditions only. The third condition still has to be examined in carp; however in murrel (Jamaluddin *et al*, 1989) it was shown that under GnRH stimulation there was an influx of extracellular Ca^{2+} from the culture medium into the gonadotrophs.

All together, our results suggest a role for extracellular Ca^{2+} in basal and GnRH-stimulated GtH release in carp. Since the lack of Ca^{2+} in the perifusion medium and the replacement of Ca^{2+} by Mn^{2+} caused total inhibition of GnRH action, we suggest that in carp, Ca^{2+} probably serves as the sole second messenger in GnRH action, while in other groups of vertebrates Ca^{2+} is one of several mediators acting in synergy, to give the complete biological response. Further studies are needed to verify this hypothesis since recent findings demonstrate that arachidonic acid metabolism participates in mediating GnRH-stimulated GtH release in the goldfish (Chang *et al*, 1989). The participation of different types of calcium channels in this process is currently under investigation.

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REFERENCES

Adams TE, Wagner TOF, Sawyer HR, Nett TM (1979) GnRH interaction with anterior pituitary. II. Cyclic AMP as an intracellular mediator in the GnRH activated gonadotroph. *Biol Reprod* 21, 735-747

Benoist L, Le Dafniet M, Rosztejn WH, Besson J, Duval J (1981) Gonadotropin release by gonadotrophs incubated with gonadotropin-releasing hormone is independent of intracellular cAMP accumulation. *Acta Endocrinol* 97, 329-337

Blake CA (1978) Changes in plasma luteinizing hormone – releasing hormone and gonadotropin concentration during constant rate intravenous infusion of luteinizing hormone – releasing hormone in cyclic rats. *Endocrinology* 102, 1043-1050

Borges JLC, Scott D, Kaiser DL, Evans WS, Thorner MO (1983) Ca^{++} dependence of gonadotropin – releasing hormone – stimulated luteinizing hormone secretion: *in vitro* studies using continuously perifused dispersed rat anterior pituitary cells. *Endocrinology* 113, 557-562

Bremner WJ, Paulsen CA (1974) Two pools of luteinizing hormone in the human pituitary: evidence from constant administration of luteinizing hormone – releasing hormone. *J Clin Endocrinol & Metab* 38, 811-819

Breton B, Weil C (1973) Effets d'extraits hypothalamiques de carpe et de LH/FSH-RH de synthèse sur la sécrétion d'hormone gonadotrope c-GTH *in vivo* chez la carpe *(Cyprinus carpio* L). *CR Acad Sci Paris Ser D* 277, 2061-2064

Breton B, Kann G, Burzawa-Gerard E, Billard R (1971) Dosage radio-immunologique d'une hormone gonadotrope de carpe (Cyprinus carpio L). *CR Acad Sci Paris Ser D* 272, 1515-1517

Breton B, Motin A, Kah O, Le Menn F, Geofre S, Precigoux G, Chambolle P (1984) Dosage radio –immunologique homologue d'un facteur hypothalamique de stimulation de la fonction gonadotrope hypophysaire de Saumon – sGnRH. *CR Acad Sci Paris Ser D* 299, 383-388

Chang JP, MacKenzie DS, Gould DR, Peter RE (1984) Effect of dopamine and norepinphrine on *in vitro* spontaneous and gonadotropin – releasing hormone – induced gonadotropin release by dispersed cells or fragments of the goldfish pituitary. *Life Sci* 35, 2027-2033

Chang JP, Freedman GL, De Leeuw R (1989) Participation of arachidonic acid metabolism in gonadotropin-releasing hormone stimulation of goldfish gonadotropin release. *Gen Comp Endocrinol* 76, 2-11

Conn PM, Rogers DC, Sandhu FS (1979) Alteration of the intracellular calcium level stimulates gonadotropin release from cultured rat anterior pituitary cells. *Endocrinology* 105, 1122-1127

Conn PM, Kilpatrick D, Kirshner N (1980) Ionophoretic Ca^{++} mobilization in rat gonadotropes and bovine adrenomedullary cells. *Cell Calcium* 1, 129-133

Conn PM, Marian J, McMillian M, Stern J, Rogers D, Hamby M, Penna A, Grant E (1981) Gonadotropin – releasing hormone action in the pituitary: A three step mechanism. *Endocr Rev* 2, 174-184

Conn PM, McArdle CA, Andrews V, Huckle WR (1987) The molecular basis of gonadotropin releasing hormone (GnRH) action in the pituitary gonadotrope. *Biol Reprod* 36, 17-35

Crim LW, Cluet DM (1974) Elevation of plasma gonadotropin concentration in response to mammalian gonadotropin releasing hormone

treatment in the male brown trout as determined by radioimmunoassay. *Endocr Res Commun* 1, 101-110

Gonnet F, Prunet P, Tonon MC, Dubourg P, Kah O, Vaudry H (1988) Effect of osmotic presure on prolactin release in Rainbow trout: *in vitro* studies. *Gen Comp Endocrinol* 69, 252-261

Gorospe WC, Conn PM (1987) Agents that decrease gonadotropin releasing hormone (GnRH) receptor internationalization do not inhibit GnRH-mediated gonadotrope desensitization. *Endocrinology* 120, 222-229

Harris CE, Staley D, Conn PM (1985) Diacylglycerols and protein kinase C: Potential amplifying mechanism for Ca^{**} mediated gonadotropin releasing hormone – stimulated luteinizing hormone release. *Mol Pharmacol* 27, 532-536

Hopkins CR, Walker AM (1978) Calcium as a second messenger in the stimulation of luteinizing hormone secretion. *Mol Cell Endocrinol* 12, 189-208

Jalabert B, Bry C, Szollosi D, Fostier A (1973) Étude comparée de l'action des hormones hypophysaires et steroides sur la maturation *in vitro* de ovocytes de la Truite et du Carassin (Poissons téléostéens). *Ann Biol Anim Biochim Biophys* 13, 59-72

Jamaluddin MD, Banerjee PP, Manna PR, Bhattacharya S (1989) Requirement of extracellular Calcium in fish pituitary gonadotropin released by gonadotropin hormone-releasing hormone. *Gen Comp Endocrinol* 4, 190-198

Levavi-Sivan B, Yaron Z (1989) Gonadotropin secretion from perifused tilapia pituitary in relation to gonadotropin-releasing hormone, extracellular calcium, and activation of protein kinase C. *Gen Comp Endocrinol* 75, 187-194

Limor R, Ayalon D, Capponi AM, Childs GV, Naor Z (1987) Cytosolic free calcium levels in cultured pituitary cells separated by centifugal elutriation: Effect of gonadotropin-releasing hormone. *Endocrinology* 120, 497-503

MacKenzie DS, Gould DR, Peter RE, Rivier J, Vale WW (1984) Response of superfused goldfish pituitary fragments to mammalian and salmon gonadotropin-releasing hormones. *Life Sci* 35, 2019-2026

Marian J, Conn PM (1979) Gonadotropin-releasing hormone stimulation of cultured pituitary cells requires calcium. *Mol Pharmacol* 16, 196-201

Ovichinnikov Yu A (1985) Ion channels: Structure and function. *Biochem Soc Symp* 46, 103-137

Peter RE, Nahorniak CS, Sokolowska M, Chang JP, Rivier JE, Vale WW, King JA, Millar RP (1985) Structure-activity relationships of mammalian, chicken and salmon gonadotropin releasing hormones *in vivo* in goldfish. *Gen Comp Endocrinol* 58, 231-242

Rasmussen H, Barret PQ (1984) Calcium messenger system: An integrated view. *Physiol Rev* 64, 938-984

Reuter H (1983) Calcium channel modulation by neurotransmitters, enzymes and drugs. *Nature (Lond)* 301, 569-574

Ribeiro L, Ahne W, Lichtenberg V (1983) Primary culture of normal pituitary cells of carp (*Cyprinus carpio* L) for the study of gonadotropin release. *In vitro* 19, 41-45

Rubin RP (1982) *Calcium and Cellular Secretion*. Plenum Press, New York

Sokolowska M (1982) The influence of luteinizing hormone releasing hormone (LHRH) on serum gonadotropin levels and oocyte maturation in female carp (*Cyprinus carpio* L) *Comp Biochem Physiol* 73a, 273-278

Stern JE, Conn PM (1981) Perifusion of rat pituitaries: Requirements of optimal GnRH-stimulated LH release. *Am J Physiol* 240, 504-509

Van Der Kraak G, Lin HR, Donaldson EM, Dye HM, Hunter GA (1983) Effect of LH-RH and des-Gly^{10} [D–Ala^6] LH–RH–ethylamide on plasma gonadotropin levels and oocyte maturation in adult coho salmon *(Oncorhynchus kisutch). Gen Comp Endocrinol* 49, 470-476

Weil C, Hansen P, Hyam D, Le Gac F, Breton B, Crim LW (1986) Use of pituitary cells in primary culture to study the regulation of gonadotropin hormone (GtH) secretion in rainbow trout: Setting up and validating the system as assessed by its responsiveness to mammalian and salmon gonadotropin releasing hormone. *Gen Comp Endocrinol* 62, 202-209