Involvement of voltage-dependent calcium channels (VDCC) in the action of GnRH on GtH release in common carp (Cyprinus carpio L): comparison with K⁺ action

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Summary — The involvement of different types of voltage-dependent calcium channels (VDCC) in the stimulatory action of GnRH (in comparison with K⁺) on maturational gonadotropin (GtH) release was investigated using superfused carp pituitary cells. The action of these 2 stimulants was not modified either by D600 or nifedipine (drugs blocking L-type of VDCC). Cadmium (Cd²⁺), which blocks all types of VDCC indifferently, provoked a dose-dependent stimulation of GtH secretion. Cd²⁺ action was not altered by addition of sGnRH in any of the doses. Similar results were obtained using K⁺ as a secretagogue, but only the highest dose of Cd²⁺ (200 μmol/l) was able to completely block K⁺ action. Low doses (0.1 and 1 μmol/l) of the L-type VDCC activator BAY-K8644 did not change basal GtH secretion and had no effect on sGnRH-stimulated GtH secretion. Surprisingly, doses (10 μmol/l and higher) of BAY-K8644 evoked dose-dependent inhibition of GtH secretion. On the other hand, a higher concentration (20 μmol/l) of nifedipine provoked a stimulation of GtH release.

Our results indicate that the stimulatory action of GnRH and K⁺ involves activation of a certain type of cadmium-sensitive VDCC (probably T- or N-type VDCC) whereas dihydropyridine and diphenylalkylamine sensitive VDCC (L-type VDCC) does not participate in this phenomenon. The inhibitory action of BAY-K8644 and, on the other hand, the stimulatory action of nifedipine indicate that L-type VDCC probably play a role in other physiological pathways regulating GtH release in carp.

GnRH / GtH / calcium channel / carp

Résumé — Implication de canaux calciques dont l'action dépend du voltage (VDCC) dans l'action du GnRH sur la libération de la GtH chez la carpe commune (Cyprinus carpio L): comparaison avec l'action de K⁺. Le rôle de différents types de canaux calciques dépendants du voltage (VDCC) dans l'action du GnRH sur la stimulation de la sécrétion de gonadotropine (GtH) par des cellules dispersées d'hypophyse de carpe a été étudié dans un système de périfusion. Le mécanisme d'action du GnRH a été comparé à celui du potassium (K⁺). L'action de ces 2 facteurs n'est modifiée ni par le D-600 ni par la nifedipine qui bloquent les VDCC de type L. Le cadmium (Cd²⁺) qui bloque indifféremment tous les VDCC induit une stimulation dont l'intensité dépend de la sécrétion du GtH, qui n'est pas modifiée en présence de GnRH et ce quelle que soit la concentration de Cd²⁺. Des résultats comparables sont obtenus avec K⁺, mais seules de fortes concentrations de Cd²⁺ (200 μmol/l) bloquent l'action de K⁺. De faibles doses de BAY-K8644, activateur des canaux de type L, ne modifient ni la sécrétion basale de GtH, ni l'action du GnRH, alors que de fortes doses (10 μmol/l) induisent une inhibition dont l'intensité dépend de la sécrétion de GtH. Par ailleurs la nifedipine à 20 μmol/l stimule elle-même la sécrétion de GtH. Ces résultats montrent que l'action stimulante du

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INTRODUCTION

It is well established that calcium ions (Ca\(^{2+}\)) play the role of second messenger in GnRH action in mammals (Conn et al., 1987; Huckle and Conn, 1988). There are more and more reports indicating that GnRH action on GtH release is also a Ca\(^{2+}\)-dependent process in fish (Jamaluddin et al., 1989; Levavi-Sivan and Yaron, 1989; Mikolajczyk et al., 1990). There is a general agreement that in higher vertebrates (birds and mammals), Ca\(^{2+}\) influx through voltage dependent calcium channels (VDCC) is responsible for prolonged LH secretion under GnRH stimulation. However, the spike phase of the secretory response to GnRH is independent of Ca\(^{2+}\) influx through VDCC (Hansen et al., 1987; Chang et al., 1986, 1988; Smith et al., 1987; Davidson et al., 1988; Smith et al., 1989). There could be an influx of Ca\(^{2+}\) through channels other than VDCC, the so-called receptor operated channels (ROC) (Conn et al., 1987; Davidson et al., 1988; Smith et al., 1989) or mobilization of Ca\(^{2+}\) from intracellular stores (Chang et al., 1986; Hansen et al., 1987). In fish literature there is also some controversy about the participation of VDCC in GnRH action. Jamaluddin et al. (1989) observed in murrel a dose-dependent inhibition of GnRH-stimulated GtH secretion by D-600 (VDCC blocker) present in the culture medium. On the other hand, Van Asselt et al. (1989) found that D-600 had no effect on spontaneous and GnRH-stimulated GtH release in African catfish, while such an inhibition was observed when nifedipine (another class of VDCC blockers) was used.

We have shown an extracellular Ca\(^{2+}\) dependence of GnRH action on GtH release in carp (Mikolajczyk et al., 1990). The aim of the present study was to compare the action of sGnRH with K\(^{+}\) (elevated K\(^{+}\) evokes depolarization of cell membrane and activates VDCC) and to find out which type of VDCC participates in the stimulatory action of these 2 secretagogues on GtH release in carp.

MATERIALS AND METHODS

Animals

Experiments were conducted for 2 consecutive years (1988 and 1989) during the natural reproductive period of carp ie from April to July. Sexually mature female carp breeders weighing 5.5–11.5 kg obtained from the Heyman Fish Farm were kept in natural ponds 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 experiments. They were exposed to a controlled photoperiod (16L: 8D) and fed ad libitum with pelleted food (Aqualim).

Cell preparation and perifusion system

Cell preparation and the perifusion system were as previously described (Mikolajczyk et al., 1990). Briefly, pituitaries (1 pituitary for each perifusion) were dispersed using collagenase.
(0.1%) (Boehringer Mannheim) in a medium
(MEM-Eagle, Gibco) buffered with 15 mmol/l
Hepes and 9 mmol/l sodium bicarbonate and
supplemented with 1% BSA (RIA grade, Sigma).
The mixture was incubated at 18 °C for 6 h, and
derision was achieved mechanically. Cells
were harvested by centrifugation, washed twice,
mixed with Bio-Gel P-2 (Biorad) and placed in
thermoregulated perfusion columns (± 10⁶ cells
per column). Five columns could be perfused at
the same time. The cells were perfused first for
18 h at a flow rate of 4 ml/h with the above medi-
urn supplemented with 2% of a serum substi-
tute, Ultroser-SF (IBF), penicillin (100 U/ml) and
streptomycin (10 µg/ml) (Gibco). The ultroser
and antibiotics were then withdrawn and the
flow rate was gradually increased up to 15 ml/h.
After 90 min of perfusion at a flow rate 15 ml/h,
the first pulse of the drugs was administered.
Fractions were collected every 7.5 min before
drug administration and every 2.5 min during
and 15 min after drug application. Details con-
cerning each perfusion are given in the figure
legends.

Drugs

Salmon GnRH (sGnRH) (Bachem), veratridine
(Sigma) and cadmium chloride were dissolved
directly in the perfusion medium prior to use.
Methoxyverapamil (D-600), nifedipine (Sigma)
and Bay-K8644 (RBI) were dissolved in ethanol
and then in the perfusion medium. The amount
of ethanol in the medium never exceeded
0.15%. The same amount of carrier was always
present in the perfusion medium passing
through control columns and had no measurable
effect on GTH release. In experiments in which
the effect of K+ depolarization was tested, the
amount of NaCl in the test medium was reduced
accordingly so that the total concentration of KCl
and NaCl remained constant.

GTH determination and calculations

GTH levels were determined using a specific RIA
developed by Breton et al (1971).

Profiles of GTH secretion are presented as a
mean percentage of the basal GTH secretion lev-
el (bl). Basal level of GTH release (100%) was
calculated as the mean of the 4 points (frac-
tions) directly preceding first drug application.
Fluctuations of GTH levels during the period di-
rectly preceding all drug administration never ex-
ceeded 20% of the basal GTH secretion level.
Fluctuations of GTH levels greater than 20%
were considered as significant. Differences in
GTH secretion rate between the control and ex-
perimental columns during the stimulation peri-
od were calculated using Student's t-test for
variation analysis.

RESULTS

Effect of organic VDCC blockers on
GnRH- and K+ stimulated GTH release

As shown in figure 1A, two 15 min pulses
of sGnRH (10⁻⁷ mol/l) evoked in the con-
trol columns a significant increase in GTH
secretion (300% bl). In the experimental
columns, the infusion of 2 pmol/l of VDCC
blocker – D-600 (interfering mostly with L-
type VDCC) had no effect on sGnRH-
stimulated GTH release. A ten times higher
(20 µM) concentration of D-600 had no ef-
teffect on sGnRH action (fig 1B) either.

At 2 and 20 µmol/l concentrations, nifedi-
pine, a specific L-type VDCC blocker had
no effect on sGnRH-stimulated GTH release (448 and 310% bl) when adminis-
terated in experimental columns in compari-
son with the control columns receiving
sGnRH alone (432 and 350% bl) (fig 2). During the infusion of 20 µmol/l of nifedi-
pine a significant increase (P < 0.01) in
GTH secretion was observed (300% bl).

A 12.5-min application of KCl (60 mM of
K+) resulted in a sharp increase (342 and 218% bl) in GTH secretion (fig 3 and data
not shown). The amplitude and profile of the
secretory response of the cells to ele-
vated K+ was similar to that caused by
sGnRH (figs 1, 2 and 5). No significant dif-
ferences were observed between the control columns receiving pulses of K+ (60 mmol/l) and the experimental columns receiving K+ in the presence of nifedipine (2 pmol/l) or D-600 (2 gmol/l) (fig 3).

**Effect of Cd2+, an inorganic VDCC blocker, on spontaneous, sGnRH– and K+-stimulated GtH release**

A 30-min administration of graded doses of CdCl2 (20, 100 and 200 µmol/l of Cd2+) resulted in a dose-dependent increase in GtH secretion (80, 110 and 170% bl respectively) (fig 4). In the case of higher concentrations of Cd2+ (100 and 200 µmol/l), the profile of GtH secretion exhibited a characteristic biphasic response: first phase during the presence of Cd2+ in the perfusion chamber and second phase after Cd2+ withdrawal (figs 4, 5A and 6). When sGnRH (10−7 mol/l) was infused during the application of Cd2+ (20 and 200 µmol/l) no changes in GtH secretion were obtained in comparison with the control
columns receiving Cd\textsuperscript{2+} alone (fig 5A). To check if Cd\textsuperscript{2+} had any side effect on sGnRH action (experiment presented in fig 5B) a similar pulse of sGnRH (alone) was applied 60 min after Cd\textsuperscript{2+} withdrawal after joint administration of Cd\textsuperscript{2+} (20 \mu mol/l) and sGnRH (10\textsuperscript{-7} \mu mol/l). This resulted, in both the experimental and the control columns, in the stimulation of GtH secretion, having a similar potency in a manner similar to earlier experiments.

The results presented in figure 6 show that 60 mM of K\textsuperscript{+} modified (P < 0.05) a secretory response of the cells to 100 \mu mol/l concentration of Cd\textsuperscript{2+} in comparison with the control columns, while no differences were observed between the control and experimental columns during K\textsuperscript{+} stimulation when 200 \mu mol/l of Cd\textsuperscript{2+} was applied.

**Effect of organic activators of VDCC on GtH release**

Veratridine (an organic membrane depolarizer) at a wide range of concentrations (0.1 to 10 \mu mol/l) had no effect on GtH release (data not shown); a weak increase in GtH secretion (30–50% bl) was observed when much higher doses were used (50 and 100 \mu mol/l respectively) (fig 7). The administration of BAY-K8644 (dihydropyridine, specific activator of L-type VDCC) in concentrations ranging from 0.01 to 1 \mu mol/l caused no changes in GtH secretion (data not shown). BAY-K8644 at a concentration of 0.1 \mu mol/l also had no effect on sGnRH-stimulated GtH secretion (data not shown). When BAY-K8644 was applied in concentrations of 10, 20 and 40 \mu mol/l it
resulted in dose-dependent inhibition of GtH secretion—28, 34 and 45% bl respectively (fig 8).

DISCUSSION

In recent years significant progress has been made in detailed characterization of the mechanism of GnRH action on gonadotropin release, mainly in rats and chickens; however there is more and more data concerning the mechanism of GnRH action in other groups of vertebrates, especially in fish (Levavi-Sivan and Yaron, 1989; Chang et al, 1989; Jamaluddin et al, 1989; Van Asselt et al, 1989; Mikolajczyk et al, 1990). It was shown in mammals, birds, and recently in fish that an increase in intracellular Ca2+ concentration is an essential step in GnRH action and that Ca2+ plays the role of second messenger in GnRH action (Conn et al, 1987; Huckle and Conn, 1988). We also proposed such a role for extracellular Ca2+ in GnRH action on GtH release in common carp (Mikolajczyk et al, 1990). The aim of the present study was to determine which type of calcium channel is responsible for Ca2+ influx during GnRH stimulation. The results presented here indicate that the stimulatory action of K+ on GtH release resembles GnRH action, indicating that the mechanism of GnRH action may involve the activation of voltage-dependent calcium channels (VDCC). It has been shown (Nowycky et al, 1985) that in neuronal tissue, 3 types (L, N and T) of VDCC exist. Each type of channel exhibits different electrophysiological and pharmacological properties (Miller, 1987). It is also possible that in pituitary cells several types of calcium channel exist, as reported by Armstrong and Matte-son (1985) in a clonal line of rat pituitary cells. In our experiments we used drugs from 2 chemical classes as VDCC blockers: dihydropyridine (DHP) and diphenylo-
alkylamine (DPA). Nifedipine (DHP) specifically blocks the L-type VDCC (so-called DHP-sensitive). D-600 (DPA) also blocks the L-type VDCC. Since, in these experiments, nifedipine and D-600 did not inhibit either K+ or GnRH action, it is clear that these 2 secretagogues (K+ and GnRH) activate other types of VDCC than DHP- and DPA-sensitive (L-type) VDCC. To verify the role of other types of VDCC we applied cadmium ions (Cd2+) which block indifferently all types of VDCC. Surprisingly, all the doses of Cd2+ used evoked a stimulation of GtH release. In this situation, to see the effect of Cd2+ on sGnRH and K+ action we were obliged to look for a modification of Cd2+ stimulatory action by sGnRH and K+. Using sGnRH as a secretagogue it was impossible to modify the profile and amplitude of the secretory response of the cells to Cd2+ (figs 5A and 5B). However, elevated K+ significantly changed the secretory response to 20 μmol/l Cd2+ (data not shown) and to 100 μmol/l Cd2+ (fig 6). When Cd2+ was applied at a concentration of 200 μmol/l, no changes in GtH secretion were observed when K+ was introduced. When these results were combined, it was concluded that Cd2+ inhibits and blocks sGnRH and K+ action. It was however necessary to use a 10-times higher dose of Cd2+ to completely block the K+ action than was the case for sGnRH. This is not surprising since elevated K+ causes a total depolarization of the cell membrane and is probably a much stronger stimulatory factor than sGnRH. One of the possible explanations of the stimulatory effect of Cd2+ could be that different VDCC blocked by Cd2+ have a different influence on GtH release. For example, the blockade of a certain type of VDCC could inhibit GnRH and K+ action while at the same time, blockade of another type of VDCC could stimulate GtH release. This hypothesis is strongly supported by results obtained using specific drugs acting on L-type of VDCC. At low doses nifedipine and BAY-K8644 had no effect on spontaneous and GnRH-stimulated GtH release. However at higher doses (10 μmol/l and more) nifedipine (channel blocker) stimulated GtH secretion with high potency and BAY-K8644 (channel activator) inhibited GtH secretion. Combined, these results suggest that during the action of Cd2+ there was a blockade of the L-type of VDCC and, as in the case of nifedipine, there was a stimulation of GtH release. At the same time there was also a blockade of N- and T-type VDCC. In adition, a biphasic secretory response of the cells to Cd2+ also tends to support our hypothesis. Each time Cd2+ was applied, GtH stimulation was stronger at the end of the Cd2+ pulse than during Cd2+ administration. This suggests that the first phase of the response to Cd2+ was caused by a blockade of L-type VDCC and the second phase (after Cd2+ withdrawal) provoked by a rise in the blockade of, and/or the reactivation of N- and/or T-type VDCC. The fact that veratridine (an organic depolarizer) was able to stimulate GtH release only at very high doses and with very low potency indicates that this drug did not activate an exact type of VDCC responsible for GtH release. Another possible explanation of the surprising effect of NFD and BAY-K8644 could be their nonspecific action when used at a high concentration (> 10 μmol/l). Drouva et al (1988) observed an inhibition of PRL and GH secretion from rat pituitary cells evoked by 100 μmol/l BAY-K8644, whereas concentrations from 0.01 up to 10 μmol/l were stimulatory. On the other hand, other data from the same laboratory (Enjalbert et al, 1988) show that BAY-K8644 at a concentration of 100 μmol/l is less effective than at 10 μmol/l in stimulating PRL secretion but has no inhibitory effect on PRL release. Chang et al (1988) have also shown that BAY-K8644 at concentrations from 1 μmol/l to 10 μmol/l
significantly increased basal LH release and enhanced LH responses to GnRH.

Our results concerning the effect of D-600 and nifedipine differ however from results obtained by other investigators in fish. Jamaluddin et al (1989) using static pituitary cell culture found a dose-dependent inhibition of GnRH-stimulated GtH release in murrel using D-600. On the other hand Van Asselt et al (1989) using perifused catfish pituitary fragments found no effect of D-600 on GnRH-stimulated GtH secretion whereas GnRH action was inhibited by nifedipine. In our opinion such differences are provoked by different experimental approaches. It could be possible that in static cultures, only the prolonged phase of secretory response to GnRH was monitored as well as in the case of perifusion of pituitary fragments (Van Asselt et al, 1989) where fractions were rarely collected (10 min) to find any effect on the first phase of the secretory response. In any case, results obtained on pituitary fragments are not so convincing since it is impossible to determine the level of drug action and to avoid possible interactions inside whole pituitary tissue.

In mammals and birds it is well established that in the prolonged phase of LH secretion under GnRH stimulation there is an influx of extracellular Ca²⁺ via voltage-dependent calcium channels (VDCC). It was also shown that the L-type of VDCC (DHP- and DPA-sensitive) is responsible for this influx since specific L-type VDCC blockers inhibited or blocked the secretory response to GnRH (Hansen et al, 1987; Smith et al, 1987; Chang et al, 1988; Davidson et al, 1988; Smith et al, 1989). If the first (spike) phase of the secretory response to GnRH is concerned there is some controversy about the pathways as well as the sources of the Ca²⁺. Chang et al (1986), Hansen et al (1987) proposed the possible participation of Ca²⁺ mobilized from intracellular stores, whereas Conn et al (1987); Davidson et al (1988) and Smith et al (1989) found that there was an influx of extracellular Ca²⁺ via an other than VDCC, probably via so-called receptor operated channels (ROC). This second hypothesis is strongly supported by the findings of Mason and Waring (1985, 1986). They found that in bovine gonadotropes GnRH does not depolarize the cell membrane which is necessary to activate VDCC. On the contrary, Croxton et al (1988) have shown that in rat gonadotropes GnRH induces oscillatory membrane currents and this could be blocked by D-600. This finding tends to support the hypothesis of the intracellular origin of Ca²⁺ in GnRH action. These findings, together with the fact that several types of calcium channels exist could be an explanation for the striking differences among the results obtained on this subject.

CONCLUSION

The present findings indicate that GnRH action in carp is probably a voltage-dependent process involving activation of Cd²⁺ sensitive VDCC. These channels are probably T- or N-type of VDCC. DHP- and DPA-sensitive VDCC (L-type) does not participate in GnRH and K⁺ action. Activation of L-type VDCC leads to inhibition and their blockade to stimulation of GtH release. This could be part of other physiological pathways regulating GtH secretion in carp. In fish, dopamine (DA) was shown to be a gonadotropin releasing inhibitory factor (GRIF), and application of DA antagonists (pimozide, domperidone) stimulates GtH secretion and strongly potentiates the action of GnRH (Peter et al, 1986). It has been shown that neuropeptide Y (NPY) also had a direct effect on GtH secretion in fish, and that its action depended on the
stage of sexual maturity of the fish (i.e. inhibitory action in vitellogenic animals, and stimulatory action in freshly ovulated fish) (Breton et al., 1989, 1990). From our results it can be supposed that the inhibition of GtH release caused by activation of L-type VDCC, reflects the action of DA or other drugs inhibiting the secretion of GtH. It could also be speculated that Ca\(^{2+}\) serves as a second messenger for both DA and GnRH, but its different transport through the cell membrane (different types of calcium channels) and probably different intracellular Ca\(^{2+}\) receptors lead finally to a different effect on GtH release. This hypothesis is currently under investigation.

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