The effects of bicuculline (a GABA_A receptor antagonist) on LHRH-A and pimozone stimulated gonadotropin (GtH2) release in female carp (*Cyprinus carpio* L.)

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The γ -aminobutyric acid (GABA) receptor antagonist, bicuculline (BIC), LHRH-A and pimozone (a dopamine receptor antagonist, PIM) were administered to mature female carp in order to test the hypothesis that BIC, like PIM, may modulate the action of endogenous dopamine (DA) at the pituitary level. Experiments conducted in two consecutive spawning seasons have shown that BIC potentiates the LHRH-A-stimulated release of GtH2. The lack of differences in GtH2 levels between the groups stimulated with LHRH-A in the presence of PIM or BIC suggests that BIC, like PIM, modulates the action of endogenous DA at the pituitary level. These data indirectly support the idea that GABA may affect GtH2 release via DA-ergic system.

Key words: Bicuculline; GABA_A receptor; LHRH-A; Pimozone; GtH2, *Cyprinus carpio* L.

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Introduction

The experiments carried out on mammals have shown that GABA, a neurotransmitter in the central nervous system, inhibits some of the physiological processes, such as noradrenaline turnover in the medial preoptic anterior hypothalamic area, tonic LH secretion or activity of tuberoinfundibular DA neurons (Barnard *et al.*, 1987; McCormick, 1989). The most recent results demonstrated that GABA neurons are involved in the regulation of endocrine function of the anterior pituitary gland and in the feedback action of gonadal steroids on gonadotropin release (McCann and Rettori, 1988). It is generally believed that GABA inhibits prolactin (PRL) as well as gonadotropin release (Mansky *et al.*, 1982; Gudelsky *et al.*, 1983; Lamberts *et al.*, 1983; Duvilansky *et al.*, 1985; Adler and

Crowley, 1986; Morello *et al.*, 1989; Akema *et al.*, 1990); however, there are data showing stimulatory effects of GABA on PRL and gonadotropin release (Ondo, 1974; Vijayan and McCann, 1978; Lamberts *et al.*, 1983; Ramirez *et al.*, 1984; Weiner *et al.*, 1988; Masotto *et al.*, 1989).

In stickleback (Follenius, 1972), goldfish (Kah *et al.*, 1986, 1991) and African catfish (Zandbergen *et al.*, 1991), the GABA-ergic fibers are located in close contact with the glandular cells of the anterior pituitary. These data suggest that in teleosts, as in mammals, GABA is involved in the control of pituitary hormone secretion. This suggestion was recently confirmed by Prunet *et al.* (1992) who showed that GABA and baclofen (a GABA_B receptor agonist) significantly inhibit PRL secretion in rainbow trout.

Because, in mammals, GABA can interact with other neurotransmitters affecting gonadotropin secretion, like LHRH (Vijayan and

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Table 1. Design of the experiments

Group	Number of fish		First injection (-2 hr)	Second injection (0 hr)
	Exp. 1	Exp. 2		
1	8	8	SAL + VEH	SAL
2	8	8	PIM + VEH	SAL
3	8	8	SAL + BIC	SAL
4	8	8	PIM + BIC	SAL
5	8	8	SAL + VEH	LHRH-A
6	8	8	PIM + VEH	LHRH-A
7	8	8	SAL + BIC	LHRH-A
8	8	8	PIM + BIC	LHRH-A

McCann, 1978; Leranthe *et al.*, 1985), norepinephrine (Mansky *et al.*, 1982) or dopamine (DA) (Fuxe *et al.*, 1975; Lamberts *et al.*, 1983) it is possible that it acts similarly in fish. It is confirmed by recent *in vitro* and *in vivo* experiments by Kah *et al.* (1992) and Sloley *et al.* (1992) in which the indirect effect of GABA on GtH2 secretion by influence via dopaminergic neurons is suggested. It is specially important in the case of GABA interaction with a DA-ergic system, which inhibits GtH2 secretion in fish (for review, see Peter *et al.*, 1986).

Recent papers by Roelants *et al.* (1990) and Kah *et al.* (1992) on the *in vivo* treatment of carp and goldfish with GABA or its agonists present contradictory results. Because of that, the present study was undertaken in two consecutive spawning seasons in order to determine the role of GABA (using bicuculline, a GABA_A receptor antagonist) in GtH2 release from the pituitary gland of female carp injected with LHRH analogue and pimozide (PIM) (D₂ receptor antagonist).

Materials and Methods

Animals

Experiments were carried out in two consecutive spawning seasons: in May, 1989 (Experiment 1) and in May, 1990 (Experiment 2) in the Department of Ichthyobiology and Fisheries of the Agricultural Academy.

For each experiment, 64 5-year-old, mature female carp (*Cyprinus carpio* L), weighing on average 3.6 kg, were used. Fish were netted from the commercial carp ponds and placed in basins of 1.6 m³, equipped with flow-through water systems capable of exchanging four times a day. The oxygen content in the water was never lower than 4 mg/l. Fish were kept at 20°C and simulated natural photoperiod (L:D = 16:8). They were not fed during the experiments.

Drugs used in the experiments

Bicuculline (BIC)—(Sigma, St Louis, MO): a GABA_A receptor antagonist dissolved in acetyl acetate (the BIC vehicle, VEH); injected at a dose of 5 mg/kg body weight.

Pimozide (PIM)—(Jansen, Belgium): D₂ receptor antagonist dissolved in acidified physiological solution (0.6% NaCl acidified with acetic acid—100 µl in 500 ml of saline) (SAL); injected at a dose of 5 mg/kg body weight.

LHRH-A: Des-Gly¹⁰ (D-Ala⁶) LHRH ethylamide—(Sigma): dissolved in acidified physiological solution (SAL); injected at a dose of 50 µg/kg body weight.

All fish were injected intraperitoneally with the first injection at 08:00 (-2 hr) and the second at 10:00 (0 hr) according to the design presented in Table 1.

Blood sampling

Experiment 1. Blood samples (0.2 ml) were taken from the caudal vasculature at 08:00, just before the first injection (-2 hr), at 10:00, just before the second injection (0 hr), and 12, 24 and 48 hr after the second injection.

Experiment 2. Blood was sampled at 08:00, just before the first injection (-2 hr), at 09:00 (-1 hr) at 10:00, just before the second injection (0 hr) and 2, 4, 6, 12 and 24 hr after the second injection.

Gonadotropin (GtH2) levels were determined by radioimmunoassay according to Breton *et al.* (1971).

Ovarian biopsy

In experiment 2, ovarian biopsy was performed just before the first injection and before the last blood sampling (Bieniarz and Epler, 1976) in order to determine the percentage of mature oocytes (after GVBD).

Statistical analysis

GtH2 levels and the percentage of mature oocytes are given as the mean with the standard error of the mean ($\pm$ SEM). Differences between these groups were statistically analyzed using one-way analysis of variance and Duncan's test.

Results

Blood gonadotropin (GtH2) levels

Experiment 1. In the first 2 hr of the experiment, mean ($\pm$ SEM) GtH2 levels varied from

Fig. 1. Changes in blood GtH2 levels in mature female carp after BIC, PIM and LHRH-A treatment (Experiment 1). Group 1: (SAL + VEH + SAL); Group 2: (PIM + VEH + SAL); Group 3: (SAL + BIC + SAL); Group 4: (PIM + BIC + SAL); Group 5: (SAL + VEH + LHRH - A); Group 6: (PIM + VEH + LHRH - A); Group 7: (SAL + BIC + LHRH - A); Group 8: (PIM + BIC + LHRH - A). Significance: a, compared with the value in groups 1, 2 and 3; b, compared with the value in group 5; c, compared with the value in groups 1, 2, 3, 4 and 5; d, compared with the value in group 2; e, compared with the value in this group before second injection. Levels of significance: one symbol (*) $P < 0.05$; two symbols (**) $P < 0.01$.

25.1 ± 5.9 to 47.2 ± 7.9 ng/ml between the groups. The differences among groups were not statistically significant.

Twelve hours after the second injection, GtH2 levels in the groups 6 (PIM + VEH + LHRH-A), 7 (SAL + BIC + LHRH-A) and 8 (PIM + BIC + LHRH-A) were 208.9 ± 33.1 , 193.4 ± 30.5 and 243.1 ± 44.2 ng/ml respectively, and were significantly higher ($P < 0.01$) compared with groups 1 (SAL + VEH + SAL), 2 (PIM + VEH + SAL) and 3 (SAL + BIC + SAL) which have, respectively, 43.2 ± 6.7 , 44.7 ± 7.7 and 36.6 ± 5.9 ng/ml, and higher ($P < 0.01$) than before the injection. There were also statistically significant differences ($P < 0.05$) in GtH2 levels between groups 7 (SAL + BIC + LHRH-A), 193.4 ± 30.5 ng/ml and 5 (SAL + VEH + LHRH-A), 110.0 ± 11.7 ng/ml.

After 24 hr, GtH2 concentrations in groups 6 (PIM + VEH + LHRH-A) and 8 (PIM + BIC + LHRH-A) were 246.3 ± 41.3 and 259.8 ± 45.1 ng/ml, respectively, and were significantly higher ($P < 0.01$) compared with GtH2 levels in groups 1-5, and higher ($P < 0.01$) than before the injection. GtH2 levels in group 7 (SAL + BIC + LHRH-A) were 137.1 ± 25.5 ng/ml and were significantly higher ($P < 0.01$) than before injection.

Forty-eight hours after the second injection, GtH2 levels in groups 1-8 varied from 109.1 ± 11.4 to 203.4 ± 28.9 ng/ml and significant differences ($P < 0.01$) were only found between groups 8 (PIM + BIC + LHRH-A) and 2 (PIM + VEH + SAL). GtH2 levels in groups 6 (PIM + VEH + LHRH-A) and 8 (PIM + BIC

+ LHRH-A) were 185.3 ± 31.7 and 203.4 ± 28.9 ng/ml, respectively, and were significantly higher ($P < 0.01$) than before injection (Fig. 1).

Experiment 2. The mean ($\pm$ SEM) GtH2 concentration in all groups at 2 and 1 hr before the second injection varied from 3.1 ± 0.7 to 9.7 ± 1.2 ng/ml and these differences were not significant.

Two hours after the second injection, GtH2 levels in groups 6 (PIM + VEH + LHRH-A), 7 (SAL + BIC + LHRH-A) and 8 (PIM + BIC + LHRH-A) 17.0 ± 5.9 , 16.7 ± 3.4 and 18.8 ± 6.1 ng/ml, respectively, were significantly higher ($P < 0.01$) compared with these in groups: 1 (SAL + VEH + SAL), 2 (PIM + VEH + SAL), 3 (SAL + BIC + SAL) and 4 (PIM + BIC + SAL). Significant differences ($P < 0.05$) were also found between groups 7 (SAL + BIC + LHRH-A) and 5 (SAL + VEH + LHRH-A).

During the next 4 hr a gradual increase in GtH2 levels in groups 5-8 was observed. The highest levels were found 6 hr after the second injection in group 6 (PIM + VEH + LHRH-A), 47.0 ± 15.3 ng/ml and in group 8 (PIM + BIC + LHRH-A), 39.0 ± 8.9 ng/ml. At the same time (4 and 6 hr after the second injection), in groups 1 (SAL + VEH + SAL), 2 (PIM + VEH + SAL), 3 (SAL + BIC + SAL) and 4 (PIM + BIC + SAL), GtH2 levels varied from 7.0 ± 2.1 to 16.0 ± 4.8 ng/ml and were significantly lower ($P < 0.01$) compared with groups 5 (SAL + VEH + LHRH-A), 6 (PIM + VEH + LHRH-A), 7 (SAL + BIC + LHRH-A) and 8 (PIM + BIC + LHRH-A). GtH2 levels in groups 5, 6, 7

Fig. 2. Changes in blood GtH2 levels in mature female carp after BIC, PIM and LHRH-A treatment (Experiment 2). Significance: a, compared with the value in group 1, 2, 3, and 4; b, compared with the value in group 5; c, compared with the value in group 1, 2, 3, 4, and 5; d, compared with the value in this group before second injection. Levels of significance as in Fig. 1.

and 8 were also significantly higher ($P < 0.01$) than before injection.

Twelve hours after the second injection, a decrease in GtH2 levels was observed in groups 5–8; however, in groups 6 (PIM + VEH + LHRH-A) and 8 (PIM + BIC + LHRH-A) (27.0 ± 8.4 and 28.0 ± 8.1 ng/ml, respectively), they were still significantly higher ($P < 0.01$) than in groups 1–5.

Twenty-four hours after the second injection, significant differences in GtH2 ($P < 0.01$) levels were found between group 6 (PIM + VEH + LHRH-A) (35.7 ng/ml) and groups 1–5 (average 8.8 ± 2.1 ng/ml) (Fig. 2).

Oocytes maturity

Before the first injection, in the ovaries of all females on average $7.0 \pm 1.7\%$ of mature

oocytes were found. After 24 hr of experiment, the percentage of mature oocytes in females from the groups 6 (PIM + VEH + LHRH-A), 7 (SAL + BIC + LHRH-A) and 8 (PIM + BIC + LHRH-A) (28.0 ± 9.2 , 25.0 ± 5.6 and $39.0 \pm 11.2\%$, respectively) was significantly higher ($P < 0.05$) compared with the percentage of mature oocytes in females from the groups 1–5 (Fig. 3).

Discussion

Two recently published papers presented contradictory results. Roelants and co-workers (1990) demonstrated in mature female carp the inhibitory action of a GABA-ergic system on PIM- and LHRH-A-stimulated GtH2 release, using GABA biosynthesis inhibitor

Fig. 3. Changes of the percentage of mature oocytes in female carp after BIC, PIM and LHRH-A treatment (Experiment 2). Significance: a, compared with the value in group 1, 2, 3, 4, and 5. Levels of significance as in Fig. 1.

(mercaptopropionic acid) or irreversible inhibitor of GABA-transaminase (γ vinyl GABA). On the other hand, Kah and co-workers (1991) showed that *in vivo* treatment of female goldfish with 10 μ g/g of GABA stimulated GtH2 release; however, the effectiveness of the treatment depended on the stage of the ovarian maturity; in regressed or in early maturing animals, GABA caused an increase of GtH2 levels, while no effect was observed in maturing and regressing fish.

The results presented in our paper on mature female carp in two consecutive spawning seasons seem to confirm the results obtained by Roelants *et al.* (1990), because it was clearly demonstrated that administration of BIC, a GABA_A receptor antagonist, potentiated GtH2 release stimulated by PIM and LHRH-A. These results indicate that GABA inhibits GtH2 secretion in mature female carp. Moreover, the same response pattern of the gonadotrops to BIC treatment was obtained in both experiments (Figs 1 and 2) despite the fact that females used for the experiments in both spawning seasons differed very much in the initial GtH2 levels and in the sensitivity to PIM and LHRH-A stimulation.

Different bleeding times in both experiments allowed one to follow the dynamics of GtH2 release under the influence of the drugs used. In both experiments, there were no significant changes in GtH2 levels in groups 1–4. In experiment 1, in groups 6 (PIM + VEH + LHRH-A), 7 (SAL + BIC + LHRH-A) and 8 (PIM + BIC + LHRH-A), a significant ($P < 0.01$) increase of GtH2 levels was found 12 hr after the second injection. At 24 hr, the GtH2 levels were maximal in groups 6 (PIM + VEH + LHRH-A) and 8 (PIM + BIC + LHRH-A), reaching a mean of 250 ng/ml. At 48 hr after the second injection, these elevated levels in groups 6 and 8 decreased, and a significant difference ($P < 0.01$) was found only between groups 2 (PIM + VEH + SAL) and 8 (PIM + BIC + LHRH-A).

More frequent blood sampling in experiment 2 shows that the increase in blood GtH2 levels in females of groups 5–8 was observed as soon as 2 hr after the second injection ($P < 0.01$) and maximal levels at 8 hr (Fig. 2). It is interesting to note that females from group 7 (SAL + BIC + LHRH-A) had significantly higher GtH2 levels ($P < 0.05$) than females from group 5 (SAL + VEH + LHRH-A) which indicates that BIC potentiates LHRH-A action. In mammals, GABA can act directly on LHRH-producing cells due to the existence of the symmetric GABA-ergic synapses on the LHRH-producing neurons (Leranth *et al.*, 1985; Akema *et al.*, 1990). Thus, it is possible that, in our experiments, the blockade of endogenous

GABA by BIC administration diminished its inhibitory action on GnRH-producing cells. It is also possible that the inhibition of GtH2 release by GABA takes place at the level of the pituitary gland, because GABA-ergic synapses were found in close contact with gonadotropic cells of goldfish pituitary (Kah *et al.*, 1986).

Interpreting the results obtained in our experiments, it is important to consider the effects of GABA on the synthesis and release of endogenous DA. Roelants and co-workers (1990) have shown no effect of GABA on spontaneous or LHRH-A-stimulated GtH2 release; however, the inhibitor of GABA biosynthesis together with LHRH-A and PIM stimulated GtH2 release. In our experiment, there were no effects of BIC either on LHRH-A or PIM and LHRH-A stimulated GtH2 release [GtH2 levels in groups 6 (PIM + VEH + LHRH-A) and 8 (PIM + BIC + LHRH-A) were not significantly different]. On the other hand, the lack of differences between groups 6 (PIM + VEH + LHRH-A) and 7 (SAL + BIC + LHRH-A) indicates that the effects of PIM or BIC on LHRH-A-stimulated GtH2 release are similar and that BIC stimulates the action of PIM, acting probably on the DA-ergic system. This observation is additionally confirmed by the fact that there is no synergism in PIM and BIC action; joint administration of PIM with BIC and with LHRH-A (group 8) caused no higher GtH2 release compared with PIM + LHRH-A (group 6) or BIC + LHRH-A (group 7). It could be that the applied dosages of PIM and BIC were high enough to activate the DA-ergic system (each of them separately). However, the influence of GABA on DA-ergic system does not seem to be as strong as the action of PIM, because the elevated levels of GtH2 after BIC and LHRH-A treatment were observed up to 12 hr and, after PIM, for a longer period of time (see groups 6 and 8 in the experiment 1).

The present results suggest that, besides its direct action on GnRH-producing cells, GABA may modulate the inhibitory effect of endogenous DA on GtH2 secretion. As was shown in goldfish by Kah *et al.* (1992) GABA has no direct effect on the gonadotrophs, because there were no effects of GABA on GtH release from dispersed pituitary cells, either in static incubation or in cell column perfusion. In the case of *in vitro* incubation of pituitary slices, containing both GABA and GnRH terminals, stimulatory effects of GABA on GnRH release were observed. The absence of the direct action of GABA on the gonadotrophs in the goldfish do not support, according to the authors, the presence of GABA receptors on the GtH cells in goldfish and indirect action of GABA on GtH release by stimulation of GnRH release.

Despite certain differences in the results of the experiments obtained on female carp by Roelants and co-workers (1990) and these presented in the recent paper, it is possible to state that in mature female carp, GABA inhibits GtH2 secretion at the time of spawning. It was also confirmed by analysis of ovarian samples taken at the beginning and at the end of experiment 2. The results show that the highest percentage of mature oocytes was found in groups 6, 7 and 8 (Fig. 3) in which the highest levels of GtH2 were observed. In female goldfish at the same state of ovarian maturity, GABA does not change GtH2 secretion, but an increase was observed in early maturing animals or in regressed fish (Kah *et al.*, 1991).

The experiments presented in our paper show the involvement of GABA-ergic system in GtH2 release from the pituitary gland of mature female carp. However, the explanation of the results needs more data, especially because of the discrepancy in the results obtained in fish at different reproductive stages, suggesting modulatory effects of steroids on GABA-ergic neurons.

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