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NALTREXONE STIMULATES LH SECRETION *IN VITRO* FROM THE COMMON CARP (*CYPRINUS CARPIO* L.) PITUITARY GLAND

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ABSTRACT. The effects of naltrexone on the *in vitro* luteinizing hormone (LH) secretion from whole pituitary glands as well as from dispersed pituitary cells of common carp (*Cyprinus carpio* L.) in primary cell culture were studied. The perifusion of whole pituitary glands with naltrexone at a concentration of 10^{-4} M caused a significant increase of LH secretion compared to the control. This finding suggests that opioid peptides can indirectly affect LH secretion at the pituitary level, probably through the nerve terminals containing GnRH and/or DA present in the pituitary. In the perifusion of dispersed pituitary cells with naltrexone at concentrations of 10^{-6} or 10^{-4} M, significant increases of LH levels were found. Similar results were observed in static culture of dispersed carp pituitary cells: after 4 or 24 hours of culture in the medium containing naltrexone at concentrations of 10^{-6}, 10^{-4} or 10^{-2} M, a dose-dependent, statistically significant increase of LH secretion to the culture medium was found. The results from dispersed pituitary cell perifusions or cell culture with naltrexone indicate that this antagonist directly affects the pituitary cells and stimulates LH release from carp gonadotrophs.

Key words: COMMON CARP (*CYPRINUS CARPIO*), *IN VITRO*, LH, NALTREXONE

INTRODUCTION

The influence of the endogenous opioid peptides on gonadotropin secretion in mammals is well documented (Grossman et al.1981, Leadem and Karla 1985, Gopalan et al. 1991). Initially, an endogenous opioid system was shown to modulate reproductive processes by acting at the hypothalamus level (Ferin et al. 1982, Bonavera et al. 1994, Walsh and Clark 1996), but further studies demonstrated their local regulatory effects at the pituitary level (Cacicedo and Sanchez-Franco 1985, Blank et al. 1986). The *in vitro* inhibition of LH release from bovine pituitary cells was observed after the administration of met-enkephalin (Chao et al. 1986). In cultured rat pituitary cells opioid peptides (β-endorphin and morphine) also directly decrease both basal and gonadotropin-releasing hormone (GnRH) stimulated LH secretion (Cacicedo and Sanchez-Franco 1985, Blank et al. 1986, Barb et al. 1990). These studies suggest that intrapituitary opioid peptides could exert a paracrine regulatory effect on gonadotroph cell function.

There is little information about the role of opioid peptides in the control of pituitary function and reproduction in fish, although their presence in the brain, hypothalamus and pituitary gland has been demonstrated in many species such as goldfish *Carassius auratus* (Follénius and Dubois 1978), rainbow trout *Oncorhynchus mykiss* (Dubois et al. 1979, Hunter and Baker 1979), European eel *Anguilla anguilla* (Carter and Baker 1980), chum salmon *Oncorhynchus keta* (Kawauchi et al. 1985), bogue *Boops boops* (Vallarino, 1985) and common carp *Cyprinus carpio* (Follénius and Dubois 1979a, b, Hon and Ng 1986). In addition, specific opioid receptors have been described in the brain of goldfish (Pert et al. 1974, Nishimura and Pasternak 1982), catfish *Ictalurus nebulosus* (Rosenblum and Callard 1988) and trout *Salmo trutta trutta* (Bird et al. 1988).

A few *in vivo* studies on fish (Rosenblum and Peter 1989, Sokolowska et al. 2002 a, b) with the use of an opioid receptor antagonist (naloxone or naltrexone) demonstrated that the secretion of LH, similarly as in mammals, is modulated by endogenous opioids. It is not clear if they affect the pituitary cells directly or if they act *via* GnRH and/or DA neurones at the hypothalamus level. In the *in vitro* studies Rosenblum and Peter (1987) and Cheng (1996) demonstrated that the administration of naloxone increased and DAGO - μ-opioid receptor agonist suppressed GnRH release from goldfish pituitary fragments.

Since in teleost fish the hypothalamo-hypophyseal portal system is not present and the hypophysiotrophic neurones penetrate the *pars distalis* of the pituitary and terminate on the pituitary cells (Ball 1981), *in vitro* investigations on the effects of opioids on gonadotropin secretion are necessary to explain the level and the site of the action of the opioids in this process. However, the results of *in vitro* experiments depend on the experimental model. The use of the whole glands does not eliminate the influence of the opioids on GnRH and/or dopamine-containing neurones, which in turn affect LH release, while the use of dispersed pituitary cells demonstrates the direct effects of the tested opioids on LH secreting cells.

Naltrexone, an opioid receptor antagonist, was used in the present study to determine its effect on the *in vitro* LH secretion from perifused whole pituitary glands as well as from dispersed pituitary cells of carp (*Cyprinus carpio* L.) in primary cell culture.

MATERIAL AND METHODS

The experiments were conducted on sexually mature, four- and five-year-old male and female carp from the Fisheries Research Station of the University of Agricul-

ture in Krakow-Mydlniki. The fish were raised in natural ponds and prior to the experiment in May or June transferred to the laboratory flow-through basins (volume 2 m^3) for at least three days. They were kept at 20°C and exposed to a simulated, natural photoperiod.

PERIFUSION OF WHOLE PITUITARY GLANDS

The fish were anaesthetized with 2-phenoxy-ethanol (0.3 ml l^{-1} of water) before decapitation and pituitary removal. The collected glands were rinsed several times with ice-cold BSS Cortland mineral medium, placed in the perifusion columns on preswollen gel (Biogel P-2, Biorad, England) and perifused at a flow rate of 20 ml h^{-1} with the medium for 90 minutes. After this time the basal level of LH secretion was established. Four 5-minute fractions were collected with a fraction collector (Fractionsammler MM 10, Germany) before each pulse of naltrexone. Then, during each of the 20-minute-long naltrexone pulses (10^{-8}, 10^{-6} or 10^{-4} M), fractions were collected every 2 minutes. After the pulse, three 5-minute-long fractions were collected. Between the pulses the pituitary glands were rinsed with medium for 60 minutes (Fig. 1). Four columns, two control columns perifused with medium and two experimental ones perifused with medium containing naltrexone, were used in one experiment. Each experiment was repeated three times.

CELL PREPARATION FOR PERIFUSION AND CELL CULTURE

The pituitary cells were prepared according to the technique previously described for trout (Veil et al. 1986) and adapted to carp (Mikolajczyk et al. 1990). In brief, after

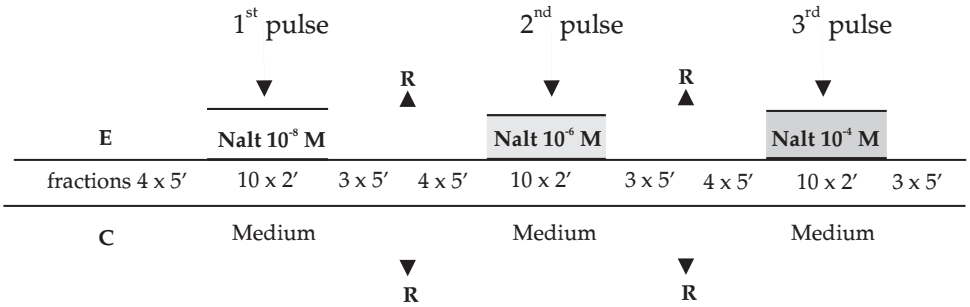

Fig. 1. The design of the perifusions of whole pituitary gland and dispersed pituitary cells (E - experimental columns, C - control columns, R - rinsing with the perifusion medium, 5′ - fractions collected every 5 minutes, 2′ - fractions collected every 2 minutes).

decapitation the pituitary glands were excised and quickly placed in sterile, ice-cold medium (MEM-Eagle, SIGMA Chemical Co., USA) buffered with 15 mM l^{-1} Hepes (SIGMA Chemical Co., USA) and 9 mM l^{-1} sodium bicarbonate. The medium had an osmotic pressure of 275 mOsm kg^{-1} and a pH of 7.7 (the values characteristic for common carp plasma). The pituitary glands were chopped into small pieces and subjected to dispersion for 6-8 hours at 20°C in a medium containing 0.1% (wt/vol.) collagenase (Boehringer Manheim GmBH, Germany) and 1% BSA (SIGMA Chemical Co., USA). Enzymatic dissociation was then completed mechanically by aspirating the fragments 7-10 times into a 10 ml syringe with a metal needle (0.1 × 10 cm). The cells were harvested by centrifugation (200 g) for 10 min at 20°C and washed twice with the pre-incubation perifusion medium which consisted of 2% serum substitute (Ultroser SF, Sepracor S.A., France) and 1% antibiotic-antimycotic (Sigma Chemical Co., USA). Cell counting and cell viability tests were performed with a Thoma's hemocytometer. After dispersion, 86-94% of living cells were obtained. Then the cells were resuspended in the pre-incubation medium and transferred to perifusion columns (6.5-7.5 × 10^5 live cells/column) on preswollen Bio-gel. For the first 16 hours the cells were perifused at a low flow rate (5 ml h^{-1}) with the pre-incubation perifusion medium. The medium was then replaced with the incubation medium without Ultroser SF and antibiotic and the flow rate was gradually increased (over one hour) to 15 ml h^{-1}. The fractions were collected similarly as is described for the perifusion of the whole pituitary glands (Fig. 1).

CELL CULTURES

After enzymatic dissociation and cell counting (as described above), the pituitary cells were cultured in 96-well plates (Nunc A/S, Denmark) covered with Poly-L-lysine (SIGMA Chemical Co., USA) to decrease the time necessary for cell adhesion to the plate. About 5 x 10^4 of living cells in 250 µl of pre-incubation medium were placed into each well. The plates were sealed and incubated for 48 hours at 22°C in the incubator. On the third day of culture the pre-incubation medium was replaced with the medium containing the tested concentrations of naltrexone (10^{-8}, 10^{-6}, 10^{-4} or 10^{-2} M) and the cells were incubated for 4 or 24 hours at 22°C. At the end of the incubation period the plates were centrifuged (200 g for 10 min at 22°C), the media were collected and cells were lysed for 4 hours by 0.1% Triton X-100 (SIGMA Chemical Co., USA) in the same buffer as was used in the ELISA measurement. The samples of media and the cell lysates were frozen at -20°C until LH determination by the ELISA method (Kah et al. 1989).

Profiles of LH from perifusions (experiment 1 and 2) are presented as the mean percentage ± SEM of the basal LH secretion level. The basal level of LH release was estimated as a mean of the four fractions which directly preceded drug application.

LH levels in the fractions from control and experimental columns were compared using the two-tailed Student's t test. The results of the *in vitro* cultures (LH levels and LH cell content) were analyzed by means of one factor ANOVA followed by the Duncan multiple range test at a $P < 0.05$ level of significance.

RESULTS

Experiment 1 – The effects of various concentrations of naltrexone (10^{-8}, 10^{-6} or 10^{-4} M) on LH secretion from the whole pituitary glands of male carp *in vitro*

The perifusion of the whole pituitary glands with the medium containing naltrexone at concentrations of 10^{-8} and 10^{-6} M did not significantly affect LH release in relation to the control (perifusion medium). The application of the highest naltrexone concentration (10^{-4}M) caused a significant ($P < 0.05$) increase of gonadotropin level in comparison to the control in four fractions (three of them were collected just after the pulse - Fig. 2).

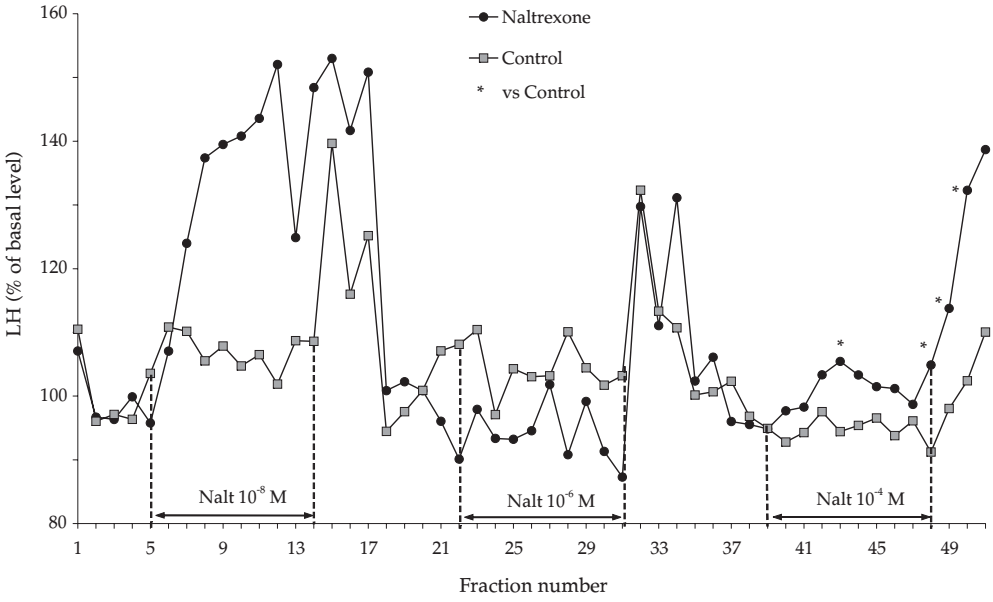

Fig. 2. Effects of various concentrations of naltrexone (10^{-8}, 10^{-6} 10^{-4} M) on LH secretion from the perifused whole pituitary glands of male carp *in vitro*.

Experiment 2 – The effects of various concentrations of naltrexone (10^{-8}, 10^{-6} or 10^{-4} M) on LH secretion from dispersed carp pituitary cells perifused *in vitro*

The perifusion of dispersed pituitary cells with the lowest of the tested concentrations of naltrexone (10^{-8} M) did not cause any significant changes in the LH secretion in relation to the control. The second pulse of naltrexone at the concentration of 10^{-6} M increased LH release in all fractions but the changes were not significantly different from LH levels found in the fractions of the control columns. Only in fraction 33, after the second pulse, was the level of gonadotropin significantly higher than in the control column. The application of the highest concentration of naltrexone (10^{-4}M) resulted in the significant ($P < 0.05$) stimulation of LH secretion (3 fractions) in relation to the control (Fig. 3).

Experiment 3 - LH release and LH content in the dispersed pituitary cells cultured with various concentrations of naltrexone (10^{-8}, 10^{-6}, 10^{-4} or 10^{-2} M)

After four hours of cell culture, a dose-dependent increase in the LH levels in the incubation medium under the influence of the tested concentration of naltrexone was found. In the incubations with Nalt 10^{-6}, 10^{-4} or 10^{-2} M, the levels of LH were significantly

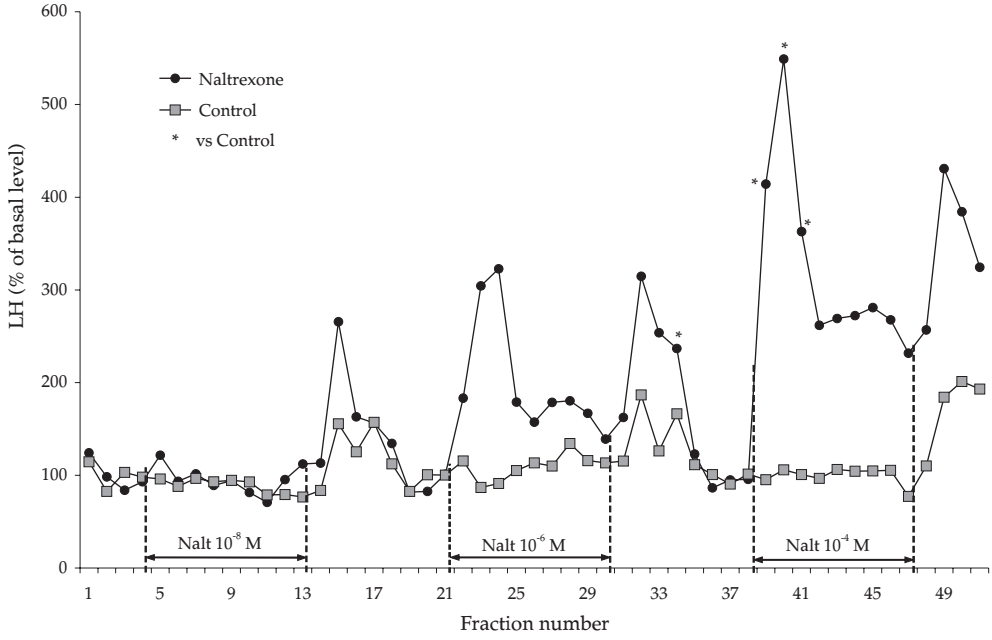

Fig. 3. Effects of various concentrations of naltrexone (10^{-8}, 10^{-6} and 10^{-4} M) on LH secretion from dispersed carp pituitary cells perifused *in vitro*.

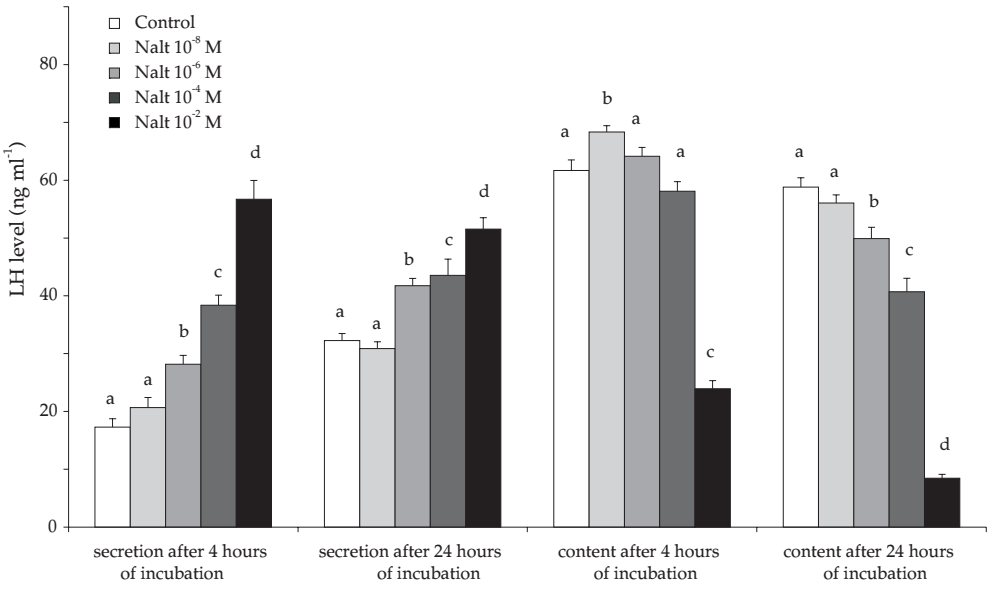

Fig. 4. LH release and LH content in the dispersed pituitary cells, cultured with various concentrations of naltrexone (10^{-8}, 10^{-6}, 10^{-4} and 10^{-2} M) during a 4 or a 24-hour incubation period. Groups bearing the same letter within the time of incubation are not significantly different ($P > 0.05$).

($P < 0.05$) higher in relation to the control (17.30 ng ml^{-1} medium) and Nalt 10^{-8} M (20.66 ng ml^{-1}). The highest, three-fold stimulation in LH secretion (56.72 ng ml^{-1} medium) was observed in the Nalt 10^{-2} M group in comparison with the control group (Fig. 4).

LH content in the cells cultured for 4 hours was significantly ($P < 0.05$) higher in the Nalt 10^{-8} M group in comparison with all other groups. In the group with the highest concentration of naltrexone (10^{-2} M), the content of LH was significantly ($P < 0.05$) lower (about two-fold) than in all the remaining groups (Fig. 4).

After 24 hours of culture, the release of LH from dispersed pituitary cells had increased significantly ($P < 0.05$) in the experimental Nalt 10^{-6}, 10^{-4} and 10^{-2} M groups in relation to the control and to the Nalt 10^{-8} M group. The highest level of LH was found in the Nalt 10^{-2} M group, and it was significantly higher than in the other groups (Fig. 4).

After 24 hours of incubation, the LH content in the cultured cells was significantly ($P < 0.05$) lower in the Nalt 10^{-6}, 10^{-4} and 10^{-2} M groups in comparison to the control

and Nalt 10^{-8} M group. The lowest LH content (8.45 ng ml^{-1}) was observed in the cells from the Nalt 10^{-2} M group, and this value was statistically significantly lower ($P < 0.05$) than the LH content in all the other groups (Fig. 4).

DISCUSSION

The few *in vivo* studies on fish (goldfish and carp) with the use of naloxone or naltrexone show that the release of LH is modulated by endogenous opioid peptides (Rosenblum and Peter 1989, Sokolowska et al. 2002a, b). To the best of our knowledge, there are also very few *in vitro* studies on the effects of opioids on the secretion of gonadotropin from fish pituitary glands. Only Rosenblum and Peter (1987) demonstrated that the incubation of hemipituitary (half of the gland) fragments with naloxone elicited an increase of GnRH release into the culture medium. A dose-related suppression of immunoreactive salmon GnRH release from pituitary fragments was observed after the administration of μ-opioid agonist (DAGO) by Cheng (1996); however, kappa and delta opioid agonists had no effects on this process. These findings suggest that opioid peptides can indirectly affect LH secretion at the pituitary level, probably through the nerve terminals of the hypothalamic neurones containing GnRH and/or DA, which are present in the pituitary fragments. These results do not, however, confirm the direct action of opioids on the gonadotroph cells. The *in vitro* secretion of adrenocortictrophic hormone from trout pituitary gland under the influence of naloxone and morphine also showed the inhibitory influence of the opiate, which is exerted on the CRF terminals within the *pars distalis* of the pituitary and not on the corticotrophs (Bird et al. 1987).

The problem of the direct influence of opioids on gonadotropin release from the gonadotrophs in mammals has already been well documented (Chao et al. 1986, Sanchez-Franco and Cacicedo 1986, Barb et al. 1990).

The experiments presented in the present paper (in which only one opioid receptor antagonist, naltrexone, was used) demonstrated that the perifusion of whole carp pituitary gland with naltrexone at a concentration of 10^{-4} M resulted in a significant increase of gonadotropin level in the perifusion medium (Fig. 2). It is possible that at this dose naltrexone affects GnRH and/or DA release from the neuron endings present in the tested glands. The response to naltrexone, measured in terms of LH release, could be resultant of activation or inhibition of both GnRH and DA systems.

The perifusion of pituitary cells after enzymatic dispersion (which eliminated the hypothalamic nerve terminals) with the same naltrexone concentrations as used for perifusion of the whole glands showed that naltrexone at 10^{-6} and 10^{-4} M caused significant increases of LH in the perifusion medium (Fig. 3). Similar results were observed in the static culture of dispersed carp pituitary cells. After 4 hours of culture in the medium containing naltrexone at concentrations of 10^{-6}, 10^{-4} or 10^{-2} M, a statistically significant, dose-dependent increase in LH release to the culture medium was observed (Fig. 4). LH levels in all groups after the 24-hour-long incubation of cells (Fig. 4) were very similar to those found in the medium after 4 hours of culture. This suggests that the release of LH under the influence of naltrexone takes place rather in the first few hours (or maybe minutes – see Fig. 2) of incubation.

The measurement of LH content in pituitary cells after 4 or 24 hours of culture with naltrexone showed that the content of gonadotropin was inversely proportional to LH release into the culture medium; along with an increase of the naltrexone concentration in the incubation medium a decrease in LH content was observed (Fig. 4). This may suggest that naltrexone affects hormone release but not its synthesis.

The results obtained with dispersed carp pituitary cells are similar to those obtained in mammals; opioid antagonist - naloxone at 10^{-6} M caused an increase of LH secretion by pig pituitary cells after 4 and 24 hours of culture *in vitro* (Barb et al. 1990). Chao et al. (1986) observed an increase in the basal release of LH from dispersed bovine anterior pituitary cells incubated with naloxone (10^{-6} M) after as few as two hours of culture. Sanchez-Franco and Cacicedo (1986) also found that naloxone (10^{-5} M) elicited an increase in LH secretion from cultured rat anterior pituitary cells.

The above-mentioned *in vitro* stimulatory action of opioid antagonists (naloxone, naltrexone) on LH secretion found in mammals and fish suggests that endogenous opioid peptides may directly regulate pituitary cell function. Chao et al. (1986) also demonstrated the antagonism between the stimulatory effect of naloxone on LH release and the inhibitory effect of opioid (met-enkephaline), which is consistent with effects exerted through opioid receptors. In the case of rat pituitary cells the effect of human β-endorphin on LH release was not reversed by naloxone (Sanchez-Franco and Cacicedo 1986). We also demonstrated the stimulatory effects of naltrexone on LH secretion in carp pituitary cell culture, which may suggest that opioid peptides directly affect *in vitro* LH release in fish. However, this conclusion should be drawn with caution as in our experiments only an antagonist, and no agonist, of opioid receptors was used. The possibility exists that in fish, similarly as in mammals, opioid

antagonists (naloxone/ naltrexone) influence gonadotropin release *via* the activation of other than typical opioid receptors (Cacicedo and Sanchez-Franco 1985). Further *in vitro* work with the combined use of opioid agonists and antagonists is necessary to determine the site of action of opioid peptides on gonadotropin release in fish.

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STRESZCZENIE

WPŁYW NALTREKSONU NA SEKRECJĘ LH Z KOMÓREK PRZYSADKI MÓZGOWEJ KARPIA (*CYPRINUS CARPIO* L.) *IN VITRO*

Celem niniejszej pracy było zbadanie wpływu naltreksonu na wydzielanie LH z całych przysadek mózgowych, jak również ze zdyspersowanych komórek przysadki mózgowej karpia. Peryfuzja całych przysadek mózgowych medium o koncentracji naltreksonu 10^{-4} M spowodowała istotny wzrost poziomu LH w medium w porównaniu z kontrolą (rys. 2). Wyniki te sugerować mogą pośrednie działanie peptydów opioidowych na sekrecje LH na poziomie przysadki mózgowej, prawdopodobnie poprzez obecne w przysadce zakończenia nerwowe, zawierające GnRH i/lub dopaminę. Istotny wzrost sekrecji LH obserwowano także ze zdyspersowanych komórek przysadki mózgowej pod wpływem naltreksonu (rys. 3). Zarówno w trakcie peryfuzji (Nalt 10^{-6}, 10^{-4} M), jak i statycznych hodowli (rys. 4) *in vitro* (Nalt 10^{-6}, 10^{-4} lub 10^{-2} M). Wyniki doświadczeń *in vitro* (peryfuzje, hodowle komórkowe) wykonanych na zdyspersowanych komórkach przysadki mózgowej wskazują, że naltrekson bezpośrednio oddziałuje na komórki przysadki mózgowej i stymuluje uwalnianie LH z gonadotropów.

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