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NICOTINE STIMULATES MATURATIONAL GONADOTROPIN (GTH_2) RELEASE FROM CARP (*CYPRINUS CARPIO* L.) PITUITARY CELLS

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Abstract—1. Perifusion of dispersed pituitary cells and pituitary cell culture was used to investigate the effects of cholinergic drugs on the secretion of maturational gonadotropin (GtH_2) in carp.

2. Nicotine strongly, and in a dose dependent manner, stimulated GtH_2 release in male and in female carp (from 10^{-8} M in the perifusion and 10^{-10} M in the cells cultures).

3. Nicotine is 10 times more active in females than in males.

4. The results suggest that in carp, nicotine stimulates GtH_2 release directly from the pituitary cells, indicating a possible involvement of a cholinergic system in the regulation of GtH_2 secretion in teleost fish.

INTRODUCTION

Maturational gonadotropin hormone (GtH_2) release in teleost fish is under complex neurohormonal and hormonal control. It is well established that in many teleost fish, including common carp (*Cyprinus carpio* L.) GtH_2 secretion is stimulated by gonadotropin-releasing hormone (GnRH) (Breton and Weil, 1973; Sokolowska, 1982; Peter *et al.*, 1986; Peter *et al.*, 1987). On the other hand, dopamine (DA) acts as an endogenous inhibitor of spontaneous GtH_2 release as well as an inhibitor of GnRH action at the pituitary level (Chang and Peter, 1983; Peter *et al.*, 1986). The GnRH–DA system is not the only one which is implicated in the regulation of GtH_2 release; recent studies have shown that in rainbow trout, goldfish and common carp neuropeptide Y (NPY) stimulates GtH_2 secretion acting indirectly (through GnRH) and also directly on the gonadotrophs (Breton *et al.*, 1989; Breton *et al.*, 1990; Peng *et al.*, 1989; Breton *et al.*, 1991). It has been recently shown that also other neurotransmitters such as GABA (Roelants *et al.*, 1990; Kah *et al.*, 1991), serotonine (Somoza *et al.*, 1988) and opioids (Rosenblum and Peter, 1989) are involved in different ways in the regulation of the GtH_2 secretion in fish. Up to now there are no data about the influence of the cholinergic system (cholinergic drugs) on the secretion of pituitary hormones in fish. In mammals acetylcholine (ACh), muscarine, nicotine and their agonists stimulate or inhibit secretion of PRL, GH, ACTH and LH mainly through activation of peptidergic (GnRH, TRH) or aminergic (DA, NE) systems (for review, see Wilson, 1979; Brown *et al.*, 1983; Gala, 1983). Specific cholinergic receptors have been found in the rat anterior pituitary gland (Schaeffer and Hsueh, 1980; Mukherjee *et al.*, 1980), but their role in the regulation of pituitary hormone secretion is unknown. In the present study, using perifusion of dispersed pituitary cells and pituitary cells culture we have investigated the effect of different cholinergic drugs on the secretion of GtH_2 from carp pituitary cells.

MATERIALS AND METHODS

Experimental animals

Experiments were conducted for two consecutive years (1989–90) during the natural reproductive period of carp. Sexually mature, 5–7 year old common carp (*Cyprinus carpio* L.) breeders (both sexes) weighing 5.5 to 11.5 kg obtained from Heyman Fish Farm were kept in natural earth ponds and then transported periodically to the laboratory. They were placed in a thermoregulated recirculating water system and acclimated at 20°C for at least one week before being used in experiments. They were exposed to controlled photoperiod (16L:8D) and fed *ad libitum* with pelleted food. Carps started to eat after 2–3 days of acclimation.

Drugs

All the drugs were purchased from Sigma, St. Louis, MO, U.S.A. (−)Nicotine, Carbachol and Tetraethylammonim chloride (TEA) were dissolved directly in the perifusion or culture medium just prior to use. The pH of the test medium with nicotine was neutralized with NaOH. Acetylcholine (ACh) and muscarine were dissolved as a 10^{-2} M stock solution in sterile saline and diluted to the working concentrations in the perifusion or culture medium just before use. Eserine was first dissolved in pure ethanol and then to the final concentration in the test

medium. The amount of ethanol never exceeded 0.01% (v/v).

Cell preparation and perifusion system

Cell preparation and the perifusion system have been previously described in detail elsewhere (Mikolajczyk *et al.*, 1990). Briefly, after enzymatical dispersion, cells were placed directly into the thermoregulated perifusion columns (max. of 5 columns could be used altogether). After equilibration time (18 hr) cells were perifused at a flow rate of 15 ml/hr. Fractions were collected every 7.5 min before and between drug application, and every 2.5 min during and 10 min after their administration. Details concerning each perifusion are given in the figure legends.

Cell culture

The protocol of carp pituitary cells dispersion was the same as for perifusion experiments. After dispersion the cells were resuspended in 2 ml of culture medium. The culture medium consisted of MEM-Eagle (Gibco laboratories) buffered with 25 mM Hepes and 9 mM sodium bicarbonate. The medium had an osmotic pressure of 295 mOsm/kg and a pH of 7.45, characteristic values of carp blood plasma. This culture medium was supplemented with 2% of serum substitute, Ultroser SF (IBF), 100 U/ml of penicillin, 10 μg/ml of streptomycin and 0.25 μg/ml of fungizone (Gibco). Cell counting and cell viability were determined in a Thoma hemocytometer. Viability of harvested cells was at least 90% as estimated by trypan blue dye exclusion. After counting, the cells were diluted to 2×10^5 cells/ml of medium and 250 μl of suspension (i.e. 5×10^4 cells) were plated in each well of 96-well plates (NUNC) precoated with 5 μg/cm^2 of poly-L-lysine (Sigma). The dishes were maintained at 24°C. After 60 hr of culture the cells were washed twice with 250 μl of ultroser and antibiotic-antimycotic free culture medium and subsequently reincubated in 250 μl of this medium with or without tested drugs. All the treatments were tested in quadruplicate and all the experiments were repeated twice. After the removal of the incubation medium, samples were immediately centrifuged for 5 min at 3°C (200 g), gently removed once again and frozen at −25°C for ELISA.

GtH_2 determination and statistics

GtH_2 levels in a culture medium were determined using a specific ELISA developed by Kah *et al.* (1989). Concentration of GtH_2 in a perifusion medium was measured using a specific RIA developed by Breton *et al.* (1971).

Profiles of GtH_2 secretion from perifusion experiments are presented as a mean percentage of the basal GtH_2 secretion level (b.l.). Basal level of GtH_2 release (100%) was calculated as a mean of the first four points (fractions) directly preceding first drug application. Fluctuations of GtH_2 levels during the period directly preceding all drug administration never exceeded 18% of the basal GtH_2 secretion level. Fluctuations in GtH_2 secretion rate greater than 20% were considered as significant. In addition, significances between initial and stimulated GtH_2 levels were checked using Duncan's multiple range test for variation analysis ($P < 0.05$). The data from cell culture experiments were analyzed using one-way analysis of variance followed by Tukey multiply range test at $P < 0.05$ level of significance.

RESULTS

Effects of different cholinergic drugs on GtH_2 secretion from perifused carp pituitary cells

As shown in Figs 1a and 1b, 12.5 min application of increasing doses of nicotine provoked a dose dependent stimulation of GtH_2 release in both sexes. In females (Fig. 1a) concentrations of nicotine of 10^{-8}, 10^{-7} and 10^{-6} M increased the GtH_2 secretion level by 39, 122 and 521% respectively. In males (Fig. 1b) nicotine was about ten times less active than in females. At concentrations ranging from 10^{-8} up to 10^{-5} M nicotine increased GtH_2 secretion level of 0, 26, 145 and 483% respectively. In both sexes, the cells began to respond to nicotine within 0–2.5 min after the beginning of the nicotine pulse. Maximal GtH_2 levels were reached within 5–7.5 min, but GtH_2 release began to decline rapidly while the secretagogue was still present in the perifusion chamber. Basal GtH_2 levels recovered immediately after nicotine withdrawal.

Application of another cholinergic drug, muscarine, in a form of 12.5 min pulses at concentrations of 10^{-8}, 10^{-7} and 10^{-6} M had no effect on GtH_2 secretion in both sexes (Figs 2a and 2b). However the highest dose (10^{-5} M) provoked a slight but significant increase in GtH_2 release of 32% of baseline in females (Fig. 2a) and 36% in males (Fig. 2b). As shown in Fig. 3, a 12.5 min application of carbachol (ACh agonist) at a wide range of concentrations (10^{-7}–10^{-5} M) had no effect on GtH_2 secretion level. Only the highest concentration (10^{-4} M) evoked a significant increase in GtH_2 secretion of 43% in females (Fig. 3a) and 45% in males (Fig. 3b).

The effects of different cholinergic drugs on GtH_2 secretion from carp pituitary cells in culture

GtH_2 release under the influence of nicotine was studied after 30 min, 1, 3, and 6 hr of incubation. The stimulatory effect of nicotine on GtH_2 release was observed in both sexes only after 30 min of incubation. Statistically significant differences ($P < 0.05$) between control and experimental groups were observed, starting from 10^{-10} M concentration of nicotine in females (Fig. 4) and 10^{-8} M in males (Fig. 5). Stimulation of the cells for longer periods (1, 3 and 6 hr) had no effect on GtH_2 secretion neither in females nor in males (data not shown). Muscarine (data not shown) and acetylcholine alone or in the presence of eserine (10^{-6} M) at different concentrations and

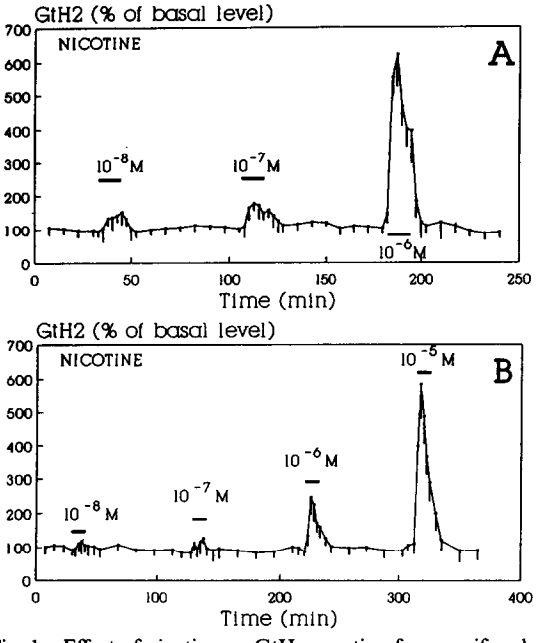

Fig. 1a. Effect of nicotine on GtH_2 secretion from perifused female carp pituitary cells. Eight columns (data from two separate experiments) received three 12.5 min pulses of nicotine in concentrations of 10^{-8}, 10^{-7} and 10^{-6} M as indicated. Mean basal GtH_2 secretion level was 26 ± 8 ng/ml. 1(b). Effect of nicotine on GtH_2 secretion from perifused male pituitary cells. Seven columns (data from two separate experiments) received four 12.5 min pulses of nicotine in concentrations of 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M as indicated. Mean basal GtH_2 secretion level was 15 ± 6.5 ng/ml.

after a different time of action had no effect on GtH_2 release in females (Fig. 6) as well as in males (Fig. 7).

DISCUSSION

It has already been shown that in mammals the cholinergic system plays a role in the control of pituitary hormones secretion (PRL, GH, LH, ACTH), mainly indirectly through activation of different neurotransmitters system (for review, see Brown *et al.*, 1983; Gala, 1983; Wilson, 1979; Hulihan-Giblin *et al.*, 1990a and b; Calogero *et al.*, 1989). Recently it was also demonstrated that cholinergic drugs act directly at the pituitary level. Thus, Rudnick and Dannies (1981) have shown an inhibitory action of ACh and muscarine on PRL secretion from rat pituitary cells in culture; however, other investigators (Campbell *et al.*, 1978) demonstrated in the same species the lack of an effect of cholinergic agonists on PRL secretion. The most recent results of Lamacz *et al.* (1989) show that, in frogs, ACh stimulated α-melanocyte-stimulating hormone release from pituitary fragments as well as from dispersed pituitary cells through activation of both nicotinic and muscarinic cholinergic receptors. The present investigations clearly show that nicotine induces an *in vitro* dose dependent stimulation of GtH_2 release directly from carp pituitary cells. These results were obtained using perifused carp pituitary dispersed cells (dynamic model) as well as primary carp pituitary cells culture (static model). The stimulatory action of nicotine on GtH_2 secretion was observed in both sexes; however its stimulatory effect was much stronger in females than in males (about 10 times in perifusion and about 100 times in static culture). It should be emphasized that nicotine action on GtH_2 release is very sharp and short. In the perifusion system, during 12.5 min of nicotine application, GtH_2 levels rise rapidly (first 5 min) and then start to decline still in the presence of the drug, reaching basal secretion level by the end of nicotine application. A similar phenomenon was observed in static culture. The stimulatory effect of nicotine was seen after a short (30 min) stimulatory period only. This indicates a possible rapid and long-lasting desensitization of nicotine receptors in carp pituitary cells. From our point of view, it is also very important that, in our experiments, the stimulatory effect of nicotine was observed using very low concentrations of this drug; 10^{-10} M in cultures and 10^{-8} M in perifusions, versus 10^{-5}–10^{-4} M, and even higher in other works.

Surprisingly, in contrast to nicotine, acetylcholine (ACh) alone or in the presence of eserine, an acetylcholinesterase inhibitor, had no effect on GtH_2 secretion from carp pituitary cells in culture as well as in perifusion (perifusion data not shown). Results obtained from the experiments with muscarine and with a nonhydrolizable ACh agonist—carbachol show their weak ability to stimulate GtH_2 secretion in

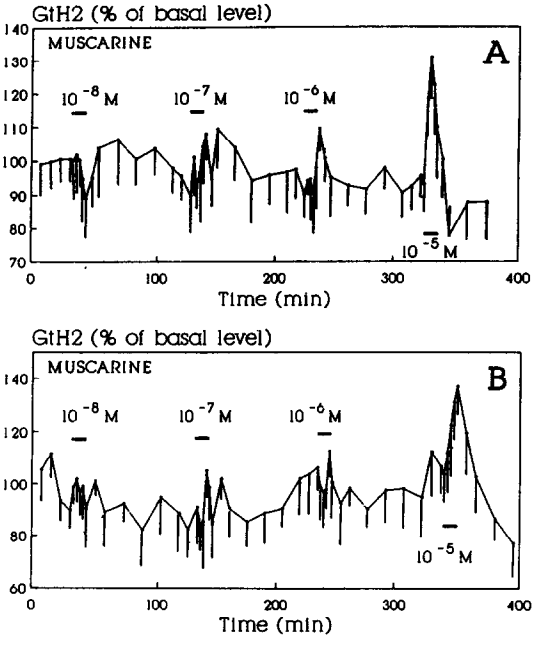

Fig. 2. Effect of muscarine on GtH_2 secretion from perifused female (a) and male (b) carp pituitary cells. Muscarine in concentrations ranging from 10^{-8} up to 10^{-5} M was administered in a form of 12.5 min pulses, as indicated. Each curve represents data from two experiments ($n = 8$ columns).

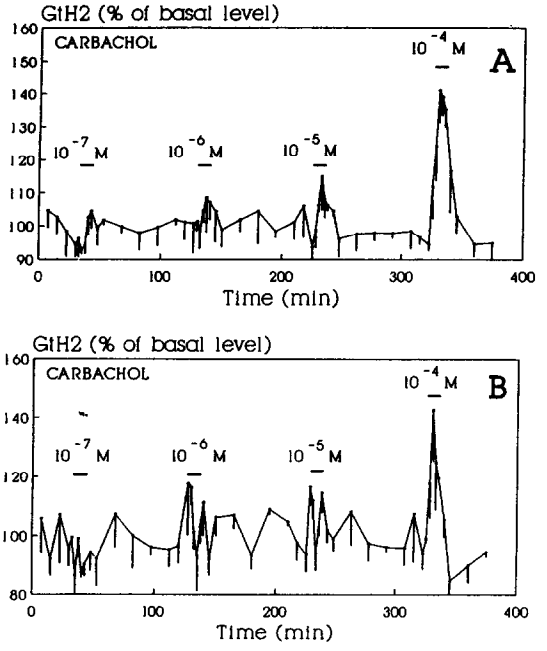

Fig. 3. Effect of carbachol on GtH_2 secretion from perifused female (a) and male (b) carp pituitary cells. 12.5 min pulses of carbachol in concentrations ranging from 10^{-7} up to 10^{-4} M were administered as indicated. Each curve represents data from two separate experiments ($n = 7$ columns).

comparison with nicotine. In the perifusion experiments, muscarine and carbachol at high concentrations (10^{-5} and 10^{-4} M respectively) significantly increased the GtH_2 secretion rate. However, their presence in a culture medium did not alter GtH_2 secretion.

Taking together all the results presented above it is possible to conclude that nicotine stimulates GtH_2 release acting directly at the pituitary cell level. Carbachol and muscarine also stimulates GtH_2 secretion but in much higher concentration than nicotine. This lets us suppose that the cholinergic system is implicated in the direct control of GtH_2 secretion

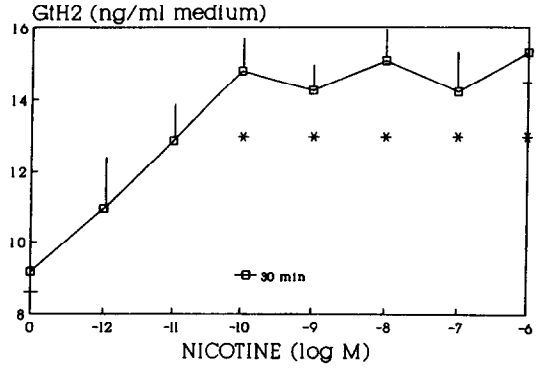

Fig. 4. Effect of nicotine on GtH_2 release from female carp pituitary cells in culture (30 min stimulation period). Data from two separate culture experiments. Each point represents the mean ± SEM ($n = 8$). *, $P < 0.05$ vs control.

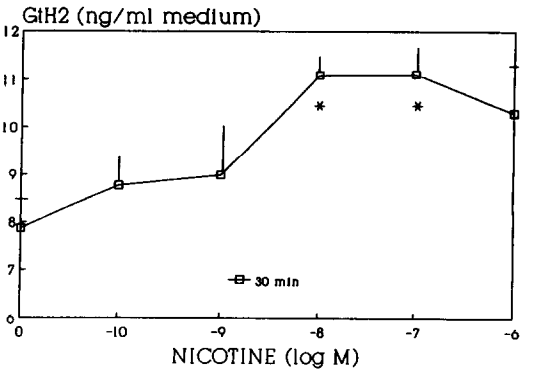

Fig. 5. Effect of nicotine on GtH_2 release from male carp pituitary cells in culture. 30 min stimulation period. Data from two separate culture experiments. Each point represents the mean ± SEM ($n = 8$). *, $P < 0.05$ vs control.

in carp. This is strongly supported by the fact that high acetylcholinesterase activity has been recently detected in carp pituitary gland and brain (Sokolowska, unpublished).

It is well known that in mammals, ACh action is mediated by two types of cholinergic receptors—muscarinic and nicotinic. Up to now there is no clear evidence for existence of the same types of ACh receptors in fish. Possible differences in the structure

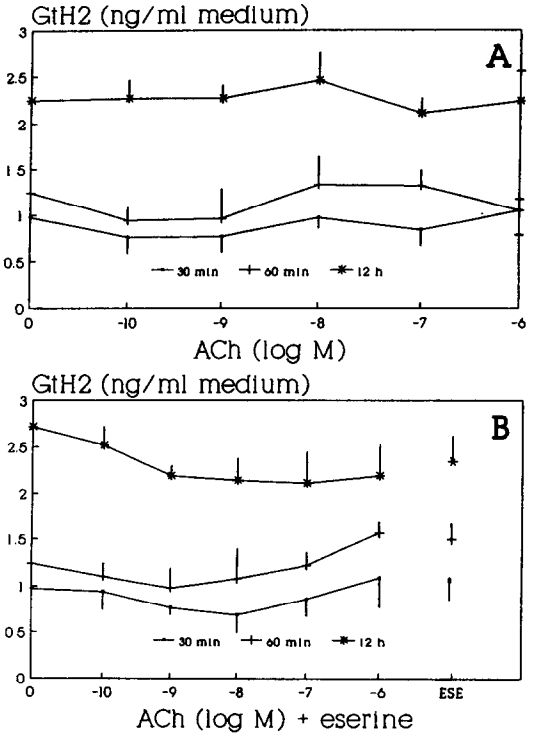

Fig. 6. Effect of acetylcholine (ACh) (A) and acetylcholine with eserine (B) on GtH_2 release from female carp pituitary cells in culture. The cells were incubated with different concentrations of ACh alone or with ACh + eserine (10^{-6} M) for 30 min, 60 min and 12 hr. ESE = eserine alone (10^{-6} M). Data from two separate culture experiments. Each point represents the mean ± SEM ($n = 8$).

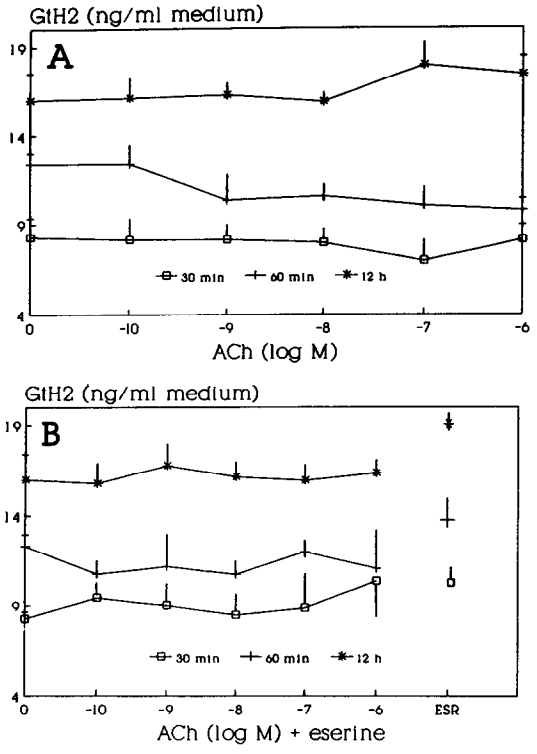

Fig. 7. Effect of acetylcholine (ACh) (A) and acetylcholine with eserine (B) on GtH_2 secretion from male carp pituitary cells in culture. The cells were incubated with different concentrations of ACh alone or with ACh + eserine (10^{-6} M) for 30 min, 60 min and 12 hr. ESE = eserine alone (10^{-6} M). Data from two experiments. Each point represents the mean ± SEM ($n = 7$).

and the activity of cholinergic receptors between mammals and fish could partially explain why ACh and carbachol were less active in the stimulation of GtH_2 secretion in comparison with nicotine. Another possible explanation could be that nicotine activates its own non-cholinergic receptors. Existence of such a type of nicotinic receptor has been already demonstrated in rat brain (Abood *et al.*, 1979; Abood *et al.*, 1980).

In summary, the present study demonstrates that nicotine strongly stimulates GtH_2 release directly from carp pituitary cells. Other cholinergic drugs such as muscarine and carbachol also exert a stimulatory effect on GtH_2 release. However their potency is much lower in comparison with nicotine. This indicates a possible involvement of the cholinergic system in the control of GtH_2 secretion in teleost fish. Consecutive studies are already undertaken to demonstrate other aspects of cholinergic control of GtH_2 secretion as well as to explain more precisely the discussed above phenomenon.

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