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GnRH SECRETING CULTURED FETAL RAT HYPOTHALAMIC CELLS DO NOT DEGRADE GnRH

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SUMMARY: The possible degradation of GnRH in the hypothalamus was investigated. Rat fetal hypothalamic cells were kept in culture for two weeks and basal and stimulated GnRH release was measured by highly sensitive RIA. These intact hypothalamic cells did not degrade GnRH during 4 hours of incubation, but a 50 % degradation occured after 24 hours incubation followed by HPLC using specifically tritium labeled GnRH or RIA. The rate of degradation or the kinetic of degradation did not change by increasing GnRH concentration in the medium or by mechanical instead of enzymatic dispersion of the cells. For comparison GnRH degradation in homogenized hypothalamic tissue and in synaptosomal preparation was measured and rapid degradation was found. Our results suggest that intact hypothalamic cells under physiological circumstances do not degrade extracellular GnRH.

The Gonadotropin -releasing hormone (GnRH) has a very central role in the regulation of reproductive functions because almost all of the regulatory parameters influence either the release of GnRH from the hypothalamus or the action of GnRH at the pituitary. As part of this regulatory mechanism it was suggested by several authors that GnRH is degraded by the hypothalamus itself (1, 2, 3, 4) and also by the pituitary (4, 5, 6, 7) in order to regulate the storage the release or the action of the neuropeptide.

GnRH besides stimulating the release of LH and FSH from the pituitary can also promote sexual behavior by a direct action on the hypothalamus and mesencephalic central gray and has been suggested to serve as a neuromodulator facilitating sexual behavior in these regions (8, 9, 10).

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It was demonstrated by several authors, that GnRH is rapidly degraded by peptidases present in both soluble and particulate fractions of the rat brain including the hypothalamus. (4, 11, 12, 20)

Changes in the activity of GnRH degrading enzyme(s) with castration and gonadal steroid injection and during the estrous cycle (1, 13) suggested that GnRH degrading enzymes might have a functional significance in controlling the amount of GnRH available for release from the hypothalamus. It was also suggested that peptidases released into the medium during in vitro incubations of the rat medial basal hypothalamus (MBH) might be important regulators of the extracellular levels of GnRH in brain tissue in vivo. (12, 14)

We have recently demostrated that intact cultured pituitary cells do not degrade GnRH (15), and now we report on our findings that functionally active, GnRH secreting cultured fetal hypothalamic cells also do not degrade GnRH.

MATERIALS AND METHODS

Synthetic GnRH was prepared in this laboratory by a Beckman-990 peptide synthesizer. ^{3}H-Pro^{9}-GnRH was prepared by catalytic tritiation from dehydro-Pro^{9}-GnRH as it has been described (16). Aliquots of the labeled hormone were freshly purified before each experiment by HPLC using conditions similar to the ones used for the analysis of incubation media.

Fetal rat hypothalamic cell culture from enzimatically dispersed cells

From the brain of about 60 17-18 days old rat fetuses the mediobasal hypothalamus (MBH) was dissected. The MBH was rostrally limited by the optic chiasm and caudally by the mamillary bodies. The lateral margins were 1 mm at each side of the midline and the depth was 1 mm. The tissues were thoroughly washed with antibiotics (Neomycin) and minced. The minced tissue was placed into 12 ml Ca^{2+}, Mg^{2+} free Tyrode solution containing 24 mg Collagenase (Worthington) and 1,2 mg DNase (Sigma) and gently stirred for 3x15 min at 37°C. After each digesting step the supernatant containing the released cells was removed, the cells were centrifuged with 600 g for 10 min. and the remaining hypothalamic (HT) pieces were further digested with the same enzyme solution. The harvested cells were washed with nutrient-medium containing 3 parts Medium-199, one part fetal calf serum (FCS, Gibco) and one part 1,2 % glucose solution, and 1 mU/ml Insulin (porcin, Sigma). One washing step included suspending of the cells, centrifugation at 600 g for 10 min., removing the supernatant and giving fresh medium. After the last washing the cells were taken up in 40 ml nutrient-medium (6-8 x 10^{5} cells/ml) and the cell suspension was distributed into 24 Falcon Petri dishes (2 ml/dish). The cells were kept in CO_2 thermostat (5 % CO_2, humidified atmosphere) at 37°C, and fresh nutrient-medium was given from the second day on each day. The experiments were carried out usually on the 10th day of culture.

Fetal rat hypothalamic cell culture from mechanically dispersed cells

All the procedures were the same as in the case of collagenase dispersed cells expect that the cells were dispersed by rigorously drawing the minced tissue in

and out in a plastic pipette. Mass tissue was allowed to settle, the supernatant containing the cells was collected and filtered through four layers of cheesecloth. The dispersion of tissue was repeated until complete. The pooled suspension of cells was centrifuged, washed and plated as above.

Hypothalamus homogenate and synaptosomal preparation

The hypothalamic homogenate and the synaptosomal fraction was prepared by Whittaker's method (17). The degradation of GnRH was followed by a quick method of. H. Berger (18) and by HPLC.

Experiments with the cultured fetal hypothalamic cells and analysis of the culture media

On the 10th day of culture the medium was removed, the cells were washed with nutrient-medium and reincubated with various agents for 4 hours. GnRH released into the medium was measured by RIA.

For degradation studies GnRH or tritiated GnRH was added to the incubation medium in final concentrations of 10^{-7} - 10^{-10} M. Aliquots of the media were taken at 0, 1, 2, 4, 6 and 24 hours and analysed for GnRH content by HPLC and RIA. Controls were incubated under identical conditions (37°C, 5 % CO_2). RIA was performed with an antiserum raised againts D-Lys^6-GnRH-BSA conjugate. The antiserum showed cross reactivity, with C terminal fragments 3-10 and 2-10 but only minor cross reactivity with shorter fragments and no binding for N terminal fragments. The sensitivity of the assay was 1 pg/GnRH/ tube. HPLC analysis of the samples was performed with a Waters HPLC system as has been described (15). The HPLC system used did resolve all possible degradation products from the ^{3}H-GnRH.

RESULTS

Fetal hypothalamic cells were kept in culture for more than two weeks and GnRH released into the medium was measured by highly sensitive RIA from the third day in culture after each day. GnRH secretion started on the sixth day and the amount of GnRH released into the medium was about the same in the forthcoming days. (200 pg/10^6 cells/24 hour.) The experiments were carried out on the 10th day of culture, because according to the morphological picture and the metabolic enzyme activities the cells became fully active on the 8th-9th day. In order to measure the specific function of the cells we investigated whether the GnRH release from these cells can be stimulated with neurotransmitters and other agents which may have a function in the regulation of GnRH release. Fig. 1. shows the effect of 10^{-7} M noradrenaline (NA), dopamine (DA), Insulin, estradiol (E_2) and testosterone (T) on GnRH release. The effect of high concentration of potassium (59 mM) which has a depolarizing effect on the plasma-membrane - was also measured. We found that both neurotransmitters strongly stimulated GnRH release from these cultured cells, K^+ also stimulated GnRH release although to a smaller extent, Insulin and the steroids had no effect on basal GnRH release.

In order to investigate whether these GnRH secreting fetal hypothalamic cells degrade GnRH or not we incubated the cells for various time periods with specif-

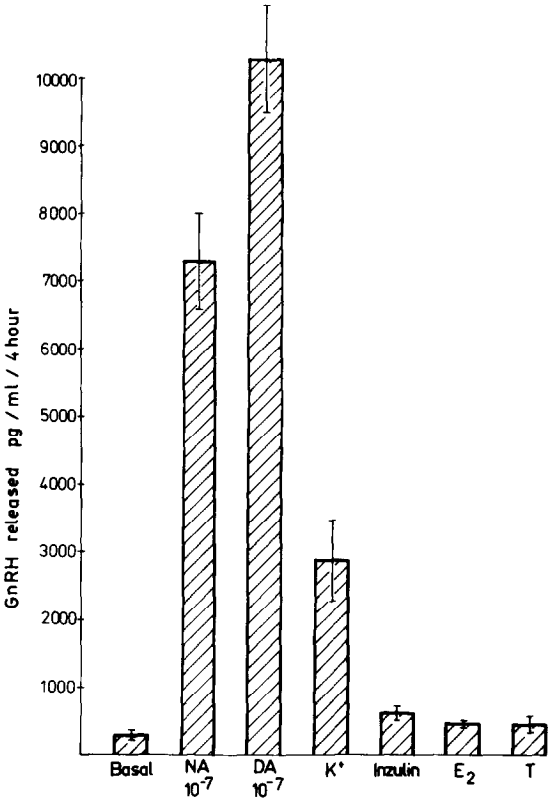

Fig.1.: GnRH released into the medium by cultured fetal hypothalamic cells during 4 hours of incubation on the 10th day of culture in the presence or absence of various agents in 10^{-7} M concentration (except K^+ which was 59 mM). GnRH content of the medium was measured by RIA. Data are the mean values of triplicate cultures ± SEM.

ically tritium labeled GnRH and aliquots of the medium was analysed by HPLC. As it is shown on Fig. 2. these intact hypothalamic cells do not degrade GnRH under the first 6 hours of incubation, but about 50 % degradation occurce after 24 hours incubation. The rate of degradation, or the kinetic of degradation did not change by increasing GnRH concentration in the medium.

To test whether a membrane bound peptidase was removed by enzymatic dispersion of the cells we measured GnRH degradation in a cell culture prepared by mechanical dispersion of the fetal hypothalamic cells.

These cells behaved entirely the same way as those which were prepared by collagenase-DNase treatment although the amount of cells obtained from one hypothalamus was much less in this case. Fig. 3. shows that these cells also do not degrade exogenous GnRH under the first 4 hours of incubation but about 40 % degradation occurce after 24 hours incubation. We also measured the change in basal,

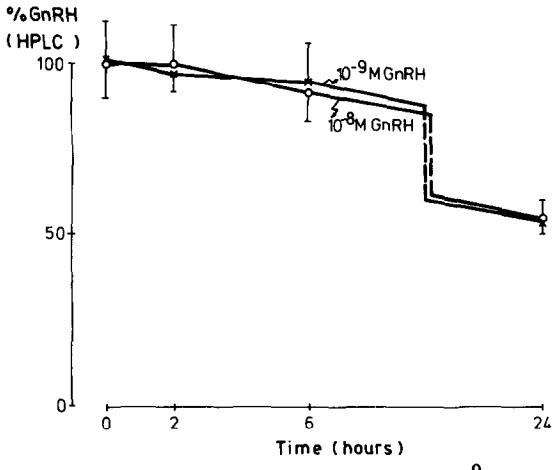

Fig.2.: GnRH content in medium during incubations of 10^{-9} M and 10^{-8} M ^{3}H-GnRH with collagenase-DNase dispersed fetal hypothalamic cell culture on the 10th day of culture as measured by HPLC. Results are expressed as percent of controls incubated without cells under identical conditions. Similar results were obtained with 10^{-10} M and 10^{-7} M GnRH.

stimulated and exogenous GnRH level by RIA. The basal GnRH level does not change during 24 hours incubation while the stimulated and the exogenously given GnRH level decreases with about 50 % after 24 hours incubation similarly as in the case of exogenously given tritiated GnRH detected by HPLC.

For comparison we measured GnRH degradation in synaptosomal preparation and in hypothalamus homogenate and we found that in both preparations GnRH was rapidly degraded as it was detected by HPLC. In our HPLC system used for detec-

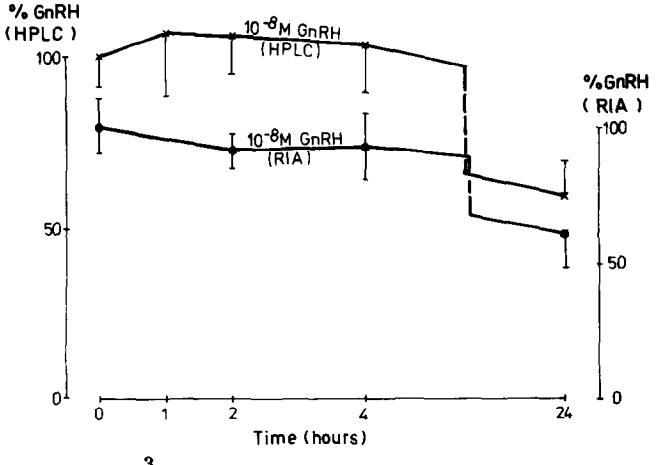

Fig.3.: GnRH (x-x) or ^{3}H-GnRH (•-•) content in medium during incubations with mechanically dispersed fetal hypothalamic cell culture as measured by HPLC (^{3}H-GnRH) and RIA (unlabeled GnRH). Results are expressed as percent of controls incubated under identical conditions without cells.

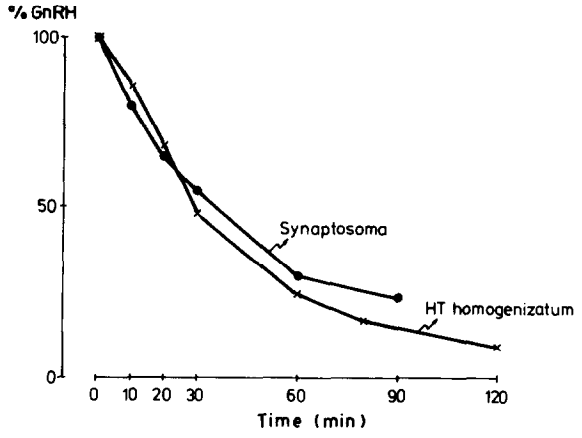

Fig.4.: The degradation of 10^{-8} M ^{3}H-GnRH by homogenized hypothalamic tissue and by a synaptosomal preparation followed by HPLC. Results are expressed as percent of controls incubated under identical conditions without cell. Similar results were obtained with non labeled GnRH followed by RIA.

tion the GnRH peak rapidly decreased while a peak close to the front (polar, small molecular weight fragments) increased simultaneously (Fig. 4).

DISCUSSION

GnRH released from the hypothalamus stimulates the LH and FSH release from the pituitary and it has been suggested that the degradation of GnRH by the hypothalamus may have a function in the regulation of reproductive functions (1, 2, 13, 20, 29).

GnRH is localized predominantly in nerve-endings (24, 25) and it have been hypothetized to act as neuromodulator or neurotransmitter i.e. to exert synaptic actions (26, 27). It also has been reported that GnRH is present in extrahypothalamic brain-locis (19, 21) to influence the firing rate of neurons variously situated in the brain (22, 23). In addition to being present in nerve-terminals and therefore available to other neurons by normal synaptic mechanisms, GnRH axons may also have access to both CSF and plasma, and the peptide may be distributed within the brain by these routes as well (19). Because all of these the regulation of extracellular GnRH level by degradation of the hormone may have a very important physiological mechanism, and it has been suggested that peptidases may be important regulators of the extracellular level of GnRH in brain tissue in vivo (30).

Previous findings reported GnRH degradation in the hypothalamus used either HT homogenates, synaptosomal preparations or incubated HT fragments. We used functionally active, GnRH secreting cultured fetal hypothalamic cells in order to

investigate whether extracellular GnRH can be degraded by these intact hypothalamic cells or not. The fate of the extracellular hormone was followed by highly specific and sensitive HPLC system. Specifically labeled ^{3}H-GnRH was used for these experiments which is structurally identical with the native hormone. The fate of GnRH released into the medium was followed by highly sensitive RIA.

We found that HT homogenate and a synaptosomal preparation also rapidly degraded GnRH which is in agreement with earlier findings in the literature. On the other hand the intact cultured fetal hypothalamic cells prepared either by enzymatic or mechanical dispersion, have not degraded GnRH at all during 4 hours of incubation. If GnRH was incubated for 24 hours with these cells about 50 % degradation of GnRH could be detected what we do not consider as a physiological mechanism for regulating GnRH level in the extracellular medium. We conclude that GnRH once secreted from the intact hypothalamic cells is not degraded by the hypothalamic cells, while we do not claim that intracellular proteases may not have a function in the regulation of intracellular GnRH level in the hypothalamic cells.

These results on the other hand together with our previous results with cultured pituitary cells (15, 28) suggest that degradation of GnRH using various tissue preparations must be studied very carefully because under the circumstances of tissue preparations nonspecific proteases can be released - for example from the lysosomes, while the intact hypothalamic and pituitary cells in culture do not degrade the extracellular hormone.

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