Opioids and cytosolic calcium in rat anterior pituitary: dynorphin preparation showed LHRH-like action due to contamination

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Summary. The effect of dynorphin A-(1-13) (Dyn A-(1-13)) and other opioids on the cytosolic free calcium concentration ($[Ca^{2+}]_i$) in rat anterior pituitary cells was examined using the fluorescent indicator fura-2. A commercial synthetic Dyn A-(1-13) preparation elevated $[Ca^{2+}]_i$. Results, which were obtained with receptor antagonists, and in LHRH receptor radioligand binding studies as well as by HPLC combined with LHRH radioimmunoassay, strongly suggest that this effect of the dynorphin preparation was due to contamination with a LHRH-like compound. Dyn A-(1-13), purified by HPLC, as well as Dyn A-(2-13), [Leu^5]enkephalin, β-endorphin, morphine, or U50,488H had no effect on $[Ca^{2+}]_i$. LHRH caused a rapid increase in $[Ca^{2+}]_i$ by about 50 nM which was blocked by the LHRH antagonist, [D-pGlu^1,D-Phe^2,D-Trp^{3,6}] LHRH.

Key words. Cytosolic free calcium; fura-2; adenohypophysis; dynorphin; contamination; luteinizing hormone-releasing hormone.

Endogenous opioid peptides regulate pituitary hormone secretion[1]. In the rat anterior pituitary gland high concentrations of dynorphin and other proenkephalin B-derived peptides are synthetized and stored within[2,3] and released from[4] gonadotrophs. The role, if any, of dynorphin in the concerted actions of the hypothalamic-pituitary-gonadal axis is, as yet, unknown.

Dynorphin may influence anterior pituitary function. The intracellular free calcium concentration ($[Ca^{2+}]_i$) in anterior pituitary cells changes coincidently with action potentials[5] and is regulated by hypothalamic neurohormones acting either through the Ca^{2+}/phosphatidylinositol messenger system[6-8] or the cAMP messenger system[9]. Dynorphin has already been shown to act, in other tissues, on receptors coupled to calcium channels[10,11], and hence dynorphin could influence $[Ca^{2+}]_i$. Therefore, we studied the effect of dynorphin A-(1-13) (Dyn A-(1-13)) and other opioids on $[Ca^{2+}]_i$ in rat adenohypophysial cells using the fluorescent indicator, fura-2[12].

Materials and methods. Male Wistar rats (200–250 g) were used. The $[Ca^{2+}]_i$ in anterior pituitary cells was measured with fura-2 as described[8,9]. The binding of ^{125}I-labeled D-Ala^6,des-Gly^{10} LHRH ethylamide (LHRHa) to crude plasma membrane fractions prepared from anterior pituitary glands was studied as described[13]. K_D values were determined in competition experiments and deduced from displacement curves as described[13]. LH and FSH release from the adenohypophysis in vitro was studied as described[4]. LH and FSH were measured by radioimmunoassays with the NIADDK kits rat reference preparations RP-2s. Dyn A-(1-13)-like immunoreactivity (IR) was measured as described[4]. LHRH-IR was measured by radioimmunoassay using an anti-LHRH-antiserum (K 29, Ferring, Kiel, FRG; 1:60,000 final dilution) and LHRH standard (Ferring, Kiel, FRG) and following a protocol as described[4]. For HPLC analysis, Dyn A-(1-13) was analyzed on a Waters µ-Bondapak C-18 reverse phase column (3.9 × 300 mm). The column was eluted with 1 M acetic acid and linear gradients of acetonitrile at a flow rate of 1 ml/min. Fractions were collected every 1 min. After evaporation the residues were redissolved and assayed for Dyn A-(1-13)-IR and LHRH-IR. The recovery of Dyn A-(1-13) was more than 95%.

Fura-2 and fura-2 acetoxymethylester were purchased from Calbiochem (Frankfurt, FRG); dynorphin A-(1-13), dynorphin A-(2-13), LHRH, LHRHa, [D-pGlu^1,D-Phe^2,D-Trp^{3,6}] LHRH, and camel β-endorphin from Sigma (Munich, FRG); [Leu^5]enkephalin from Peninsula (Belmont, CA, USA); Na^{125}I from NEN (Dreieich, FRG); [^{125}I] iodo-LHRH from Amersham (Braunschweig, FRG); morphine hydrochloride from Boehringer (Ingelheim, FRG); U50,488H was a generous gift from Dr P. Illes (Freiburg, FRG); naloxone hydrochloride was a gift from Endo Lab. (Garden City, NY, USA). Unless stated otherwise, in the present study synthetic Dyn A-(1-13) was used as supplied by the manufacturer without further purification. Values are means ± SEM. Student's t-test was used for comparisons between two means.

Results. In cells incubated in medium containing 1.3 mM Ca^{2+}, the mean resting $[Ca^{2+}]_i$ was 228 ± 8 nM (n = 18). The removal of extracellular Ca^{2+} or the addition of Ca^{2+} channel blockers decreases the mean resting $[Ca^{2+}]_i$[8,9]. A commercial synthetic Dyn A-(1-13) preparation (1 µM) produced an increase in fluorescence intensity within seconds

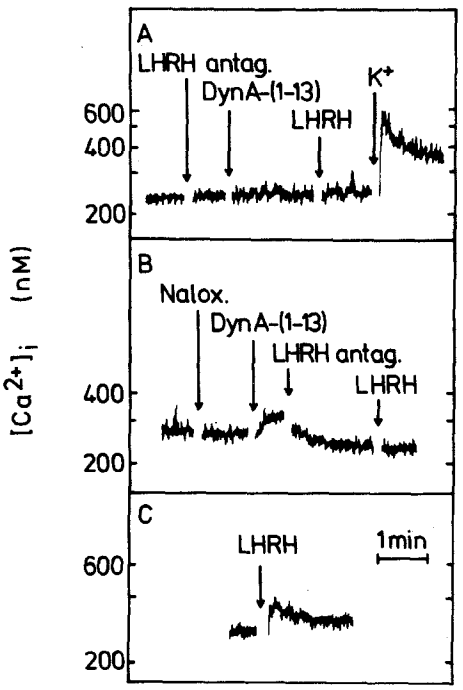

Figure 1. Effect of a LHRH antagonist and naloxone on the Dyn A-(1-13)-induced increase in cytoplasmic Ca^{2+} concentration. LHRH antag., LHRH antagonist [D-pGlu^1,D-Phe^2,D-Trp^{3,6}] LHRH (1 µM); Dyn A-(1-13) (1 µM); LHRH (10 mM); K^+ (25 nM)-induced rise in $[Ca^{2+}]_i$ was unimpaired in presence of LHRH antagonist and amounted to 289 ± 34 nM (n = 5); Nalox., naloxone (10 µM). *A*, *B* and *C* Representative tracings of 3 or 4 independent experiments each.

(fig. 1 B). $[Ca^{2+}]_i$ increased by about 60 nM to a plateau which was maintained during the observation period of about 3 min (not shown). This effect was concentration-dependent, EC_{50} being 35 nM (not shown). The opiate antagonist, naloxone (10 μM), had no effect when given alone and did not block the effect of Dyn A-(1-13) (1 μM) (fig. 1 B). Dyn A-(1-13) (100 nM) increased $[Ca^{2+}]_i$ by 47 ± 6 nM (n = 4) or 49 ± 5 nM (n = 7) in the presence or absence of naloxone (10 μM), respectively. The LHRH antagonist [D-pGlu[1],D-Phe[2],D-Trp[3,6]] LHRH (1 μM) did not affect $[Ca^{2+}]_i$ when given alone. However, it blocked the effect of Dyn A-(1-13) (1 μM) on $[Ca^{2+}]_i$ (fig. 1 A). Likewise, the increase in $[Ca^{2+}]_i$ induced by Dyn A-(1-13) (1 μM) in the presence of naloxone (10 μM) was reversed by the addition of the LHRH antagonist (1 μM) (fig. 1 B). The binding of [^{125}I]iodo-LHRHa to anterior pituitary membranes was inhibited by Dyn A-(1-13) (K_D 6.3 ± 0.4 × 10^{-6} M, n = 6) indicating an interaction with LHRH receptors. The K_D was about 700-fold higher than the respective K_D of LHRH (9.1 ± 4.2 × 10^{-9} M, n = 8). The K_D value found for LHRH is similar to that which has previously been reported [14]. Dyn A-(1-13) (1 μM) stimulated the release of LH and FSH from the anterior pituitary in vitro. LH release increased from 33.9 ± 3.3 ng/90 min (n = 8) to 83.7 ± 4.9 ng/90 min (n = 8; $p < 0.001$) and FSH release from 35.9 ± 2.2 ng/90 min (n = 8) to 100.7 ± 9.5 ng/90 min (n = 8; $p < 0.001$) upon Dyn A-(1-13) (1 μM) stimulation. In the Dyn A-(1-13) preparation LHRH-IR was found by radioimmunoassay. LHRH-IR amounted to about 30 μmol/mol Dyn A-(1-13). In HPLC analysis the LHRH-IR eluted from the column with a retention time different from that of Dyn A-(1-13) and also different from that of the LHRH standard (not shown). A preparation of Dyn A-(1-13), purified by this HPLC system, had no effect on $[Ca^{2+}]_i$ in rat anterior pituitary cells and also did not influence the K^+ (25 mM)-induced rise in $[Ca^{2+}]_i$ (not shown). The effect of other opiate receptor agonists on $[Ca^{2+}]_i$ was also studied. As shown in figure 2 [Leu[5]]enkephalin (1 μM), which is Dyn A-(1-5), did not change $[Ca^{2+}]_i$. Dyn A-(2-13) (100 nM), or the opiate active compounds, camel β-endorphin (1 μM), morphine (10 or 100 μM), or U50, 488H (1 μM), a kappa-receptor type agonist, also did not affect $[Ca^{2+}]_i$ in rat anterior pituitary cells (not shown).

LHRH (10 nM) caused a sudden increase in fluorescence intensity (fig. 1 C). The initial increase in $[Ca^{2+}]_i$ amounted to 46 ± 6 nM (n = 10). LHRH (100 nM) did not produce a greater response (45 ± 5, n = 5). The LHRH (10 nM)-caused increase in $[Ca^{2+}]_i$ was completely abolished by the LHRH antagonist, [D-pGlu[1],D-Phe[2],D-Trp[3,6]] LHRH (1 μM; not shown).

Discussion. The effect of a commercial synthetic Dyn A-(1-13) preparation on cytosolic free calcium concentration in normal rat anterior pituitary cells, found in this study, appears to be due to contamination with another biologically active peptide. The nature of the contamination remains unknown. However, it may be a potent LHRH analogue. These conclusions are based on the results which were obtained with receptor antagonists, in LHRH receptor radioligand binding studies as well as by HPLC in combination with LHRH radioimmunoassay. Dyn A-(1-13) purified by HPLC had no effect on $[Ca^{2+}]_i$. The LHRH-IR eluted in the HPLC system in a fraction different from Dyn A-(1-13) and also different from LHRH standard. This excludes the possibility of a crossreaction of authentic Dyn-(1-13) with the anti-LHRH-antiserum. It indicates a contamination of the Dyn A-(1-13) preparation with a highly potent LHRH analogue rather than LHRH itself. There are several other recent reports dealing with the problem of contamination. Abou-Samra et al.[15] found contamination of commercial synthetic atrial natriuretic factors with a gonadotropin-releasing hormone agonist. Bex et al.[16] showed that LH-releasing activity observed in a glucagon-selective somatostatin analogue was due to a contamination with a LHRH agonist, and Tannenbaum et al.[17] reported about contamination of growth hormone-releasing factor with corticotropin releasing factor. Thus, contamination of synthetic peptides appears to be of major relevance, as even minute contamination with highly active peptides, as shown in the present work, could account for apparent, new biological effects.

Like Dyn A-(1-13), purified by a HPLC system, Dyn A-(2-13), or other opiate receptor agonists such as [Leu[5]]enkephalin, β-endorphin, morphine, or the kappa-opiate receptor-selective agonist, U50,488H, did not change $[Ca^{2+}]_i$. This lack of effect does not support the view that dynorphin and other opioids, when given alone, may influence anterior pituitary function through an effect on $[Ca^{2+}]_i$. However, the present study does not exclude a change in $[Ca^{2+}]_i$ in a small subpopulation of anterior pituitary cells or regulatory influences of endogenous opioids on the adenohypophysis, which are not associated with a change in $[Ca^{2+}]_i$. Blank et al.[18] found an inhibition of LH release by morphine and endogenous opiates, possibly β-endorphin, in cultured pituitary cells.

With quin2, and in highly enriched gonadotrophs, Clapper et al.[19], Chang et al.[7], and Limor et al.[20] have reported an LHRH-induced increase in $[Ca^{2+}]_i$ of about 10–20, 70, and 90 nM, respectively. If LHRH acts only on gonadotrophs, the increase in $[Ca^{2+}]_i$ within the gonadotrophs can be estimated to be 50 nM, 120 nM, or 150 nM, respectively (based on the percentages of gonadotrophs given). In the present study using normal adenohypophysial cells, containing at the most about 10% of gonadotrophs [21], the LHRH-induced increase in $[Ca^{2+}]_i$ was about 50 nM and may, thus, be estimated to be 500 nM in gonadotrophs. This greater estimated increase in $[Ca^{2+}]_i$ upon LHRH stimulation in the present study could be explained by less Ca^{2+} buffering capacity in fura-2 than in quin2 loaded cells [12]. It suggests that the increase in $[Ca^{2+}]_i$ in rat gonadotrophs may have been underestimated so far.

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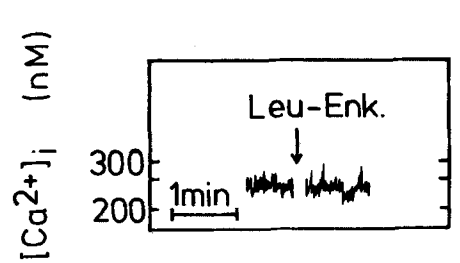

Figure 2. Effect of [Leu[5]]enkephalin on cytosolic free calcium concentration. Leu-Enk., [Leu[5]]enkephalin (1 μM). Representative tracing of 3 independent experiments.

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Thyroid hormone binds to human corpus luteum

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Summary. A high affinity, low capacity 3,5,3'-triiodo-L-thyronine (T_3) specific binding was detected in the nuclei preparation from human corpus luteal nuclei by using an in vitro thyroid hormone binding assay. The bound hormone was efficiently separated from free hormone by the use of 40% polyethylene glycol. Under standardized assay conditions of pH 8.6 at 37 °C and a 2-h incubation time, the binding in the corpus luteal nuclei was saturable with Kd 4.94×10^{-10} M with low maximum binding capacity (1.70 p mol/mg DNA). Competitive binding studies with hormone analogues indicated that T_3 binding to corpus luteal nuclei is hormone-specific. Findings indicated a direct effect of thyroid hormone on the human corpus luteum.
Key words. Thyroid hormone; human corpus luteum; triiodothyronine binding.

Thyroid has long been involved in the reproduction of vertebrates[1-7]. Thyroid hormone has been found to influence both ovarian[8,9] and testicular[10-13] functions in mammals. Hypothyroid women have irregular menstrual bleeding due to the failure in progesterone secretion[14]. Alteration in steroid hormone metabolism due to hypothyroidism can be improved by restoring the euthyroid state[15]. Although these reports suggest an influence of thyroid hormone on gonadal activity, how it does so remains unclear. Recently, we have reported high affinity and low capacity thyroid hormone binding sites in the ovarian nuclei of perch[16] implying a direct involvement of thyroid hormone in the reproduction. Thus it would be worthwhile to evaluate reproductive malfunction in hypothyroid patients provided binding sites for thyroid hormone can be detected in human ovaries. We describe here the validation of an in vitro triiodothyronine (T_3) binding assay for human corpus luteum nuclei and examine the binding activity of different thyroid hormone analogues.

Materials and methods. The minute portion of the ovary containing corpus luteum was obtained from women undergoing laparatomy for nonendocrine conditions during the luteal phase of the menstrual cycle. The average age was between 37–40 years. The procedure had the approval of the local hospital (Suri Sadar Hospital, West Bengal, India). Indications for surgery were as follows: a) menorrhagia, b) menorrhagia in fibroids, c) fibroids, d) carcinoma of the cervix and e) sterility.

Nuclei from corpus luteal cells were isolated following the method of Jackson and Chalkey[17] as described by Lawson et al.[18]. All isolation procedures were carried out near 0 °C. The corpus luteal tissue, after removal, was immediately kept in ice-cold SMNaT buffer (0.25 M sucrose, 10 mM $MgCl_2$, 50 mM $NaHSO_3$ in 10 mM Tris buffer, pH 7.5) and washed thoroughly. It was then cleaned, blotted, weighed, minced and homogenized in SMNaT buffer containing 1% (v/v) Triton X-100 using a Potter-Elvehjem type glass homogenizer. The homogenate was passed through double-layered cheese cloth and centrifuged at 2000 g in a refrigerated centrifuge. The pellet containing the crude nuclei was rehomogenized and washed thrice as above. Finally the pellet was washed twice in SMNaT buffer (without Triton X-100) to remove Triton X-100. At each step the nuclei were microscopically checked (Carl-zeiss, Jena) with aceto-orcein stain (2% orcein in 50% glacial acetic acid). The final nuclear material was found to be free of 'ghost' cell membranes or other debris and was designated as 'pure nuclear preparation'. This preparation was immediately used for receptor binding study, although it is stable at −20 °C for more than 7 days.

In vitro T_3-binding assay was based on the description of DeGroot and Torresani[19] and Darling et al.[20] with a few modifications reported earlier by us[16]. Standardization of T_3 binding assay for luteal cell nuclei preparation include optima for time, temperature, pH and polyethylene glycol (PEG) concentration, and these are described in the results. For Scatchard analysis, nuclear preparation was suspended in SMCT incubation buffer (binding assay buffer) containing 0.32 M sucrose, 3 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM DTT in 10 mM Tris buffer, pH 8.6. 80 µg of DNA (per tube) was incubated with increasing concentrations of ^{125}I-T_3 (sp. activity 180.6 µCi/µg) from 2.833 to 0.194 p mol in 500 µl of SMCT buffer at 37 °C for 2 h. DNA was estimated following the procedure of Howell[21] and also by spectral analysis as mentioned by Lawson et al.[18]. Both methods gave similar results. The amount of nuclei added in each incubation was determined by the quantity of DNA added to each tube. The incubation was terminated by placing the tubes on crushed ice for 10 min. Bound and free hormones were separated by adding chilled 40% PEG (mol. wt 6000). The tubes were kept in ice for 10 min and then centrifuged at 2500 g. Supernatant was aspirated and the pellet was counted in a Gamma Counter (ECIL, India). Nonspecific binding was determined