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Stimulation by Proopiomelanocortin-Derived Peptides of LH Release by Bullfrog Dispersed Anterior Pituitary Cells

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ABSTRACT—Labeling and immunoprecipitation experiments confirmed that three proopiomelanocortin (POMC)-derived peptides, the N-terminal peptide of POMC (NPP), joining peptide (JP) and adrenocorticotropic hormone (ACTH), were released by the bullfrog (*Rana catesbeiana*) anterior pituitary. The effects of these three peptides on luteinizing hormone (LH) release by bullfrog dispersed anterior pituitary cells were studied. NPP and ACTH, but not JP, enhanced LH release concentration-dependently. Approximately 6 hr elapsed before the gonadotrophs responded to NPP and ACTH by releasing LH, whereas their response to human GnRH (hGnRH) was faster, suggesting that the modes of action of these two peptides and hGnRH differ. These results raise the possibility that NPP and ACTH act as paracrine factors in the bullfrog pituitary to enhance LH release either directly or indirectly.

INTRODUCTION

It has been established that post translational processing of proopiomelanocortin (POMC) generates several biologically active peptides, as well as peptides with biological functions that are obscure (Hanneman *et al.*, 1989). We isolated substantial amounts of the joining peptide (JP) and N-terminal peptide of POMC that does not contain γ-melanophore-stimulating hormone (NPP) from the anterior lobe of the bullfrog pituitary (Iwamuro *et al.*, 1992), which suggested that they are products of the anterior pituitary cells, presumably the corticotrophs. These two peptides have been generally regarded as products of the intermediate, not the anterior, lobe (Eipper and Mains, 1980). The NPP thus obtained was demonstrated to have a stimulatory effect on the frog interrenal tissue (Iwamuro *et al.*, 1992). Elevation of adrenal function by NPP has also been reported in mammals (Estivariz *et al.*, 1982; Lis *et al.*, 1981) and teleosts (Takahashi *et al.*, 1985). The biological function of JP, however, remains to be determined.

Evidence is growing that, in mammals, different types of anterior pituitary cell interact with each other by producing paracrine factors (Denef *et al.*, 1989; Schwartz and Cherney, 1992). It has also been suggested that pituitary hormones act as paracrine factors in amphibians. We found in the bullfrog that prolactin enhanced gonadotropin-releasing hormone (GnRH)- induced luteinizing hormone (LH) release (Oguchi *et al.*, 1997) and that the α-subunit of glycoprotein hormones stimulated the prolactin release (Oguchi *et al.*, 1996).

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As a step toward elucidating further functions of POMC-derived peptides, we examined the effects of NPP, JP and adrenocorticotropic hormone (ACTH) on LH release by bullfrog dispersed anterior pituitary cells and found that NPP and ACTH, but not JP, enhanced LH release.

MATERIALS AND METHODS

Hormones and antisera

Bullfrog NPP (fNPP) and JP (fJP) consisting of 48 and 35 amino acids, respectively, were purified as described by Iwamuro *et al.* (1992). Bullfrog ACTH (fACTH), a 39 amino acid polypeptide, was prepared by solid-phase chemistry (Taker Chaise Co. Ltd., Shigeo, Japan), according to the amino acid sequence deduced from bullfrog POMC cDNA (Aida *et al.*, 1994). Human GnRH (hGnRH) and ovine corticotropin-releasing factor (oCRF) were purchased from the Peptide Institute (Osaka, Japan). Polyclonal anti-fACTH and anti-fJP antisera were raised in rabbits as described by Tanaka *et al.* (1992) and Iwamuro *et al.* (1993), respectively, and an anti-fNPP antiserum was raised in a female albino rabbit by the lymph node injection technique (Goudie *et al.*, 1966). Before using the latter antiserum, its specificity was confirmed by immunoblotting and immunohistochemical staining. Briefly, Western blot analysis revealed that the anti-fNPP antiserum recognized purified fNPP, but neither fJP nor fACTH, and immunostaining of the bullfrog pituitary revealed that it stained the cells similar to those stained with anti-fACTH antiserum.

Labeling and immunoprecipitation

Two distal pituitary lobes of adult bullfrogs were cut into small pieces and incubated with ^{35}S-methionine (Amersham, England) in methionine-free 70% Dulbecco's modified Eagle's medium (Gibco, Detroit, MI) containing 0.1% BSA (Fraction V, Sigma, St. Louis, MO) and oCRF (10^{-7} M) for 5 hr at 25°C. The medium was decanted, diluted with immunoprecipitation buffer (10 mM Tris-HCl, 0.1% SDS, 0.1% Triton X-100, 2 mM EDTA, pH 7.4) and immunoprecipitated with the anti-fNPP, anti-fJP or anti-fACTH antiserum, as described previously (Miura *et al.*, 1982). The immunoprecipitates were sepa-

rated electrophoretically on a 15-25% SDS-polyacrylamide gradient slab gel (Daiichi Pure Chem. Co., Tokyo, Japan), according to the method of Laemmli (1970), using Tris-tricine buffer (pH 8.3), and then visualized fluorographically (Bonner and Laskey, 1974). The individual fNPP, fJP and fACTH preparations were subjected separately to SDS-polyacrylamide gel electrophoresis in the same manner and each band was stained with Coomassie brilliant blue R (Fluka Chemicals, Germany). Molecular marker used in this experiment was the product of Daiichi Pure Chem. Co. (Tokyo, Japan).

Cell incubation and hormone assay

Adult male bullfrog dispersed anterior pituitary cells were prepared as described previously (Oguchi *et al.*, 1996), and suspended in 70% Eagle's minimum essential medium (MEM) without phenol red (Nissui Pharmaceutical, Tokyo, Japan) containing 0.1% BSA and 20 mM HEPES (Sigma, St. Louis, MO). The volume of the suspension was adjusted with this medium so that 1 ml contained 8×10^4 cells, and 2×10^4 cells in 250 μl medium were plated in each well of a 96-multiwell plate (Corning Inc., NY). Preincubation was continued for 24 hr unless otherwise stated. After preincubation, the medium was replaced with MEM containing the required test substance, fNPP, fJP, fACTH or hGnRH, and the 96-multiwell plate was incubated at 25°C in a humidified atmosphere of 95% air-5% CO_2 for various times. The medium was collected from each well, centrifuged at 800 rpm for 5 min and the supernatant was subjected to radioimmunoassay for bullfrog LH (fLH) (Oguchi *et al.*, 1997).

Statistics

The data were expressed as means ± SEM and analyzed using analysis of variance and Student's *t*-test. Differences at $P<0.05$ were considered significant.

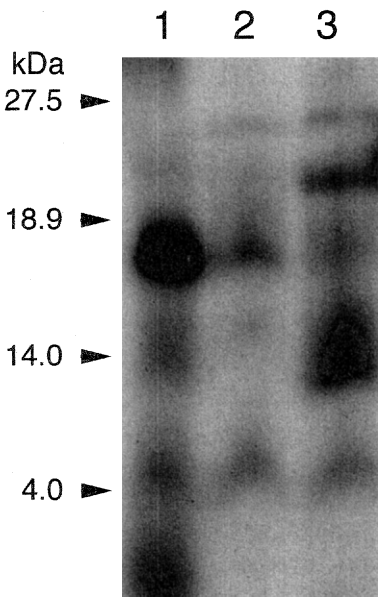

Fig. 1. Immunoprecipitation analysis of the POMC-derived peptides released by bullfrog anterior lobes. Anterior pituitaries were incubated with ^{35}S-methionine in the presence of 10^{-7} M oCRF and the media were subjected to immunoprecipitation with anti-fNPP, anti-fJP or anti-fACTH antisera, followed by SDS-PAGE and fluorography. Lanes 1, 2 and 3: immunoprecipitates with anti-fNPP; anti-fJP; and anti-fACTH, respectively.

RESULTS

In order to confirm that the anterior pituitary of the bullfrog actually released the POMC-derived peptides that were to be tested for biological activity, immunoprecipitation analysis was performed. As shown in Fig. 1, each antiserum precipitated polypeptides of varying molecular sizes. The peptides that migrated to 4-5-kDa positions were concluded to be fNPP, fJP and fACTH, as electrophoretic bands of purified fNPP and fJP and synthetic fACTH appeared at comparable positions (data not shown).

The amounts of immunoassayable LH released by the dispersed pars distalis cells into the culture media containing hGnRH, fNPP, fJP or fACTH were followed for 24 hr. As shown in Fig. 2, hGnRH stimulated LH release markedly during the initial 6 hr of incubation and moderately during the last 18 hr of the incubation period. Slight, but non-significant, enhancement by fNPP and fACTH of LH release was observed after incubation for 6hr, followed by marked and significant elevation during the next 6 hr, whereas, no stimulation of LH release by fJP was observed throughout the 24 hr incubation period.

As shown in Fig. 3, fNPP and fACTH both enhanced LH release by the dispersed pituitary cells in a concentration-dependent manner, whereas, fJP, at the concentrations examined (10^{-12}-10^{-7} M), did not stimulate LH release (data not shown). The minimum effective concentrations of fNPP and fACTH were approximately 10^{-11} and 10^{-12} M, respectively.

Experiments were conducted to ascertain whether it took

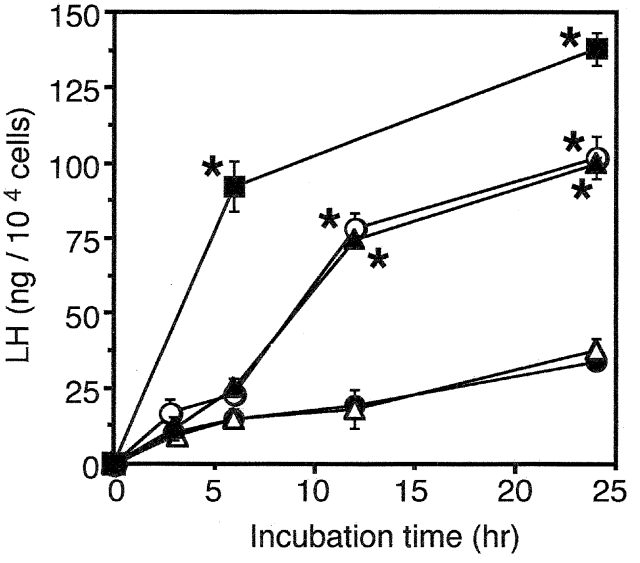

Fig. 2. Time courses of fNPP-, fJP- and fACTH-induced LH release by bullfrog dispersed pituitary cells. The concentrations of each test substance used was 10^{-7} M, and hGnRH (10^{-6} M) was used as a positive control. After preincubation for 24 hr, incubation was continued for 3, 6, 12 or 24 hr. ●, control; ▲, fNPP; △, fJP; ○, fACTH; ■, hGnRH. The values are means ± SEM (n = 6). *Significantly different from the control values ($P<0.001$).

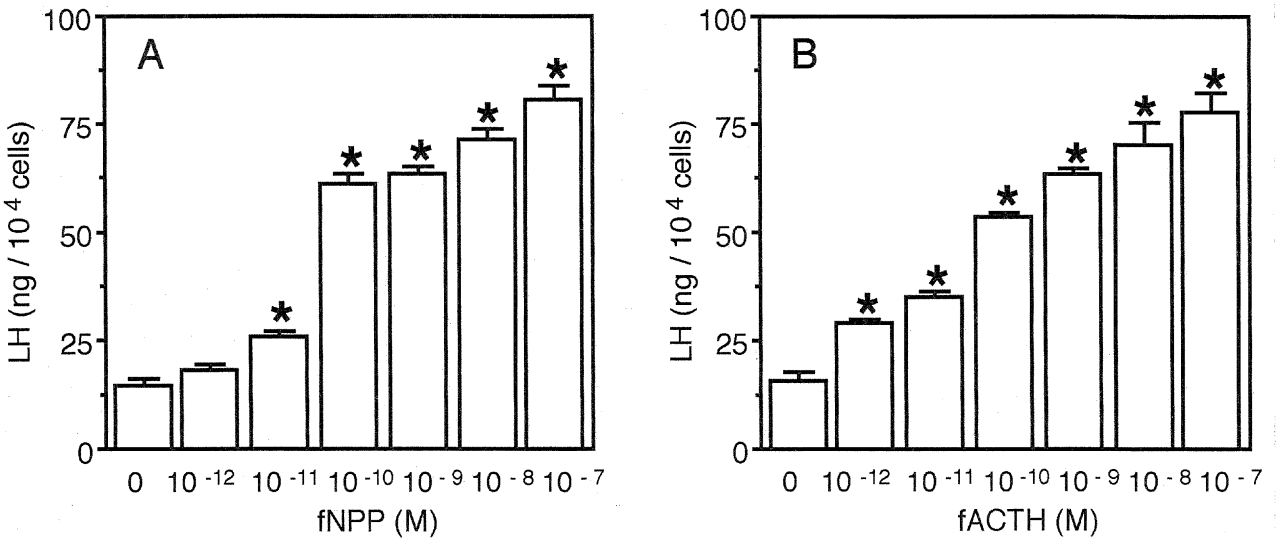

Fig. 3. Effects of various concentrations of fNPP (**A**) and fACTH (**B**) on LH release by bullfrog dispersed pituitary cells. After preincubation for 24 hr, the cells were incubated for 12 hr. The values are means ± SEM (n=6). *Significantly different from the control values (P<0.001).

Table 1. Effects of preincubation and/or incubation with fNPP and fACTH on LH release by dispersed pituitary cells

Treatment		
Preincubation	Incubation	LH (ng/10^4 cells)
None	None	12.2 ± 1.6^a
None	fNPP	13.8 ± 2.9^a
fNPP	None	50.8 ± 0.6^b
fNPP	fNPP	67.4 ± 3.1^c
None	fACTH	14.0 ± 2.2^a
fACTH	None	54.5 ± 3.3^b
fACTH	fACTH	74.6 ± 3.1^c

The cells were preincubated for 6 hr and incubated for 6 hr with various combinations of fNPP (10^{-7} M), fACTH (10^{-7} M) or medium alone and the amounts of LH released during the 6-hr incubation period were determined by RIA. The values represent the means ± SEM of 6 determinations and those with the same superscripts do not differ significantly from each other (P>0.05).

the cells longer to respond to the POMC-derived peptides than to hGnRH. The results summarized in Table 1 indicate there was a delay before the cells responded to fNPP and fACTH and started to release amounts of LH above the control levels. Cells preincubated with fNPP or fACTH and then incubated with medium only, released significantly more LH than cells preincubated with medium only and then incubated with fNPP or fACTH. Less LH was released by cells preincubated with fNPP or fACTH than by cells preincubated and incubated with these peptides, but the differences were relatively small.

DISCUSSION

The results of this study demonstrated that fNPP and fACTH, but not fJP, stimulated LH release by bullfrog dispersed anterior pituitary cells in culture. It is generally accepted that proteolytic cleavage of the N-terminal portion of POMC in the intermediate lobe of the pituitary generates NPP, γ-melanophore-stimulating hormone and JP and that the N-terminal portion of POMC is not processed in the anterior lobe (Eipper and Mains, 1980). In a previous study, we isolated substantial amounts of NPP and JP from the bullfrog anterior pituitary (Iwamuro *et al.*, 1992), which suggested that the N-terminal fragment of POMC is processed, at least, partially in the frog distal lobe to generate JP and NPP. This was confirmed by our labeling and immunoprecipitation experiments, which showed that bullfrog dispersed anterior lobe cells released NPP-, JP- and ACTH-like peptides into the culture medium.

NPP and ACTH stimulated LH release by dispersed pituitary cells and their respective minimum effective concentrations were 10^{-11} and 10^{-12} M. As the circulating concentrations of ACTH in the frog, *Rana ridibunda*, have been reported to be around 8×10^{-12} M (Vaudry *et al.*, 1975), it is possible that the released POMC-derived peptides stimulate LH release *in vivo* by acting on the neighboring gonadotrophs.

It is well known that considerable transformation of the anuran pituitary structure occurs during metamorphosis. Before metamorphosis, the dorsal side of the entire anterior pituitary is attached to the median eminence. As metamorphosis proceeds, the caudal part of the pars distalis gradually becomes detached from the median eminence and finally, is connected to the median eminence only at the rostral part by the portal vessel (Etkin *et al.*, 1965). It is noteworthy that bullfrog immunoreactive ACTH cells are localized in the rostral region (Tanaka *et al.*, 1992), which is considered to be situated upstream of the pituitary circulation. Therefore, it is quite possible that POMC-derived peptides released by ACTH cells reach the LH cells, which are distributed in the dorso-caudal region of the adenohypophysis (Tanaka *et al.*, 1991, 1992), via the pituitary circulation and enhance LH release.

We observed marked enhancement of LH release by hGnRH within 6 hr of culture, whereas it took 12 hr for fNPP and fACTH to enhance LH release indicating that the modes of action of hGnRH and these two POMC-derived peptides differ. However, whether fNPP and fACTH act indirectly on LH cells remains to be elucidated.

From our concentration-response data, the LH-releasing activity of purified fNPP was estimated to be approximately one-tenth of that of synthetic fACTH. In a previous study, we measured the amount of ACTH in the fNPP material using a radioimmunoassay (RIA) that crossreacted fully with fACTH (Jégou *et al.*, 1983; Vaudry *et al.*, 1984) and found that the residual amount of ACTH comprised less than 0.005% of the purified fNPP (Iwamuro *et al.*, 1992). Accordingly, the activity of the NPP preparation can not be accounted for by contamination with ACTH and fNPP is considered to possess intrinsic LH-releasing activity.

Bullfrog NPP is known to have a relatively high sequence homologies with NPPs of other vertebrates (Iwamuro *et al.*, 1992): it shows 100, 85 and 50% sequence homology with *Rana ridibunda* (Hilario *et al.*, 1990), *Xenopus laevis* (Martens, 1987) and human (Seidah *et al.*, 1981) NPPs, respectively. The amino acid sequences of human (Cochet *et al.*, 1982), monkey (Patel *et al.*, 1988), bovine (Nakanishi *et al.*, 1979), porcine (Boileau *et al.*, 1983), rat (Drouin *et al.*, 1985), mouse (Uhler and Herbert, 1983) and salmon (Kitahara *et al.*, 1988) JPs, deduced from their cDNA sequences, are highly variable, indicating that the structure of JP has diverged considerably during evolution. In fact, fJP exhibits 86 and 54% homology with *Rana ridibunda* (Hilario *et al.*, 1990) and *Xenopus laevis* (Martens, 1987) JPs, but only 9% homology with human JP (Chang *et al.*, 1980). In our study, fJP did not show LH-releasing activity, whereas fNPP and fACTH did. The lack of phylogenetic structural conservation of JP might explain its lack of bioactivity. In contrast, the fact that the amino acid sequences of NPP are conserved throughout the vertebrate phylum suggests that this peptide may play important roles. In fact, NPP has been shown to be involved in the regulation of adrenocortical activity in the rat (Lis *et al.*, 1981), rainbow trout (*Salmo gairdneri*) (Takahashi *et al.*, 1985) and frog (*Rana ridibunda*) (Iwamuro *et al.*, 1992). Recently, the structure of the N-terminal portion (26 amino acids) of POMC was reported to be responsible for directing POMC to the regulatory pathway (Cool *et al.*, 1995).

In conclusion, our data show that the NPP and ACTH stimulate LH release by the bullfrog anterior pituitary and raise the possibility that these peptides act on the gonadotrophs in a paracrine fashion.

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