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Effect of GnRH Antagonists on Phorbol Ester-Induced LH Release from Rat Pituitary Gonadotrophs

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Abstract. We previously reported that a blockade of GnRH receptor activation inhibited the already-initiated C-kinase pathway(s). We tried to investigate whether this finding is a general phenomenon or not. In this study, we employed three GnRH antagonists, [D-Phe^2, Pro^3, D-Phe^6]-GnRH, [Ac-D-Nal-Ala^1, D-pCl-Phe^2, D-Ser(Rha)^6]-GnRH, and [Ac-D-p-Cl-Phe^{1,2}, D-Trp^3, D-Lys^6, D-Ala^{10}]-GnRH (referred to as #1-, #2-, #3-GnRH antag., respectively). Each antagonist was examined for its potency against GnRH by analyzing its inhibitory effect on LH release from pituitary gonadotrophs as well as on the increase in the cytosolic Ca^{2+} concentration. As a result, the #1-GnRH antag. was found to be weaker than the other two compounds. Consistent with a previous study, the #3-GnRH antag. inhibited the action of TPA on LH release. However, independently of their potency as GnRH-antagonists, the two other antagonists had no inhibitory effect on TPA-induced LH release. While it is generally accepted that the C kinase pathway plays a major role in the GnRH-induced LH release, not all GnRH antagonists can inhibit LH release by blocking the already-activated C kinase system.

Key words: GnRH, GnRH-antagonist, Phorbol-ester, Pituitary gland, LH, C-Kinase, Intracellular calcium.
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IT IS WELL known that many intracellular mechanisms are involved in LH release after the binding of GnRH to GnRH receptor on the surface of plasma membrane in pituitary gonadotrophs. We previously reported that protein kinase C and calmodulin-dependent kinase participate in GnRH-induced LH release, and that either kinase system has a regulatory effect on the other [1]. This finding suggested that both kinase systems are connected by metabolic pathway(s), which transmits regulatory signals in a bi-directional way.

Beside this interaction between the two kinase systems, we also reported the interesting finding that a potent GnRH antagonist inhibited phorbol ester-induced LH release. This finding was a minor result in our previous paper, and implies that a blockade of GnRH receptor activation could inhibit the already-initiated metabolic pathway(s). In this study, we investigated whether this finding is a general phenomenon or not.

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Materials and Methods

Hormones and drugs

A GnRH antagonist, [D-Phe^2, Pro^3, D-Phe^6]-GnRH, was purchased from Peninsula Laboratories (Belmont, CA) and is referred to as a #1-GnRH antagonist in this paper. Another GnRH antagonist, [Ac-D-Nal-Ala^1, D-pCl-Phe^2, D-Ser(Rha)^6]-GnRH (referred to as a #2-GnRH antagonist in this

paper), was generously supplied by Dr. Sandow J. A potent GnRH antagonist, [Ac-D-p-Cl-Phe^{1,2}, D-Trp^{3}, D-Lys^{6}, D-Ala^{10}]-GnRH, was a gift from Dr. D. Coy, used as a representative GnRH antagonist in a previous paper by us [1], and is referred to as a #3-GnRH antagonist in this paper.

An active phorbol ester, 12-o-tetradecanoyl-phorbol-13-acetate (TPA), was purchased from Sigma Chemical Co. (St. Louis, MO). Stock solutions of 10^{-4} M GnRH (Gonadorenin acetate; Tanabe Seiyaku Co., Ltd., Tokyo, Japan) and GnRH antagonists in 0.01 M PBS were aliquoted and stored at −80°C. TPA and fura 2-AM (Calbiochem, San Diego, CA) were dissolved in dimethyl sulfoxide (Sigma Chemical Co., St. Louis, MO) at 1 mM and stored at −80°C.

Pituitary cell preparation and culture

Pituitary glands were collected after decapitation of adult female Spraque-Dawley rats (200–250 g body weight). Posterior lobes of pituitary glands were removed and anterior lobes were dispersed by a modified method of Vale *et al.* with trypsin, as previously reported [2–3]. The dispersed cells were aliquoted (2.5×10^5 cells/m*l*) into 24-well tissue culture plates (Corning, USA) and preincubated at 37°C under 5% CO_2-95% air in Earle's medium 199 (M199, GIBCO) containing 25 mM Hepes, 10% horse serum, penicillin and streptomycin.

Incubation of pituitary cells with hormones and drugs

After 3 or 4 days' culture, the cells were washed twice with 1 m*l* of Earle's medium 199 containing 25 mM Hepes and 0.3% bovine serum albumin (BSA fraction V, Sigma Chemical Co.). The stock solutions of hormones and drugs were diluted to various concentrations and cells were incubated in 1 m*l* of the medium containing hormones and drugs in quadruplicate cultures. The incubation was continued for 4 h at 37°C under 5% CO_2-95% air. At the end of the incubation, the medium was stored at −80°C until assayed for LH content by RIA.

$[Ca^{2+}]_i$ assays with Fura 2

The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in pituitary gonadotroph was investigated by fluorescence analysis after Fura 2-loading at dual excitation wavelength in a spectrofluorometer (CAF-110, JASCO Co., Ltd., Tokyo, Japan) as previously described [3].

Assay of LH and data analysis

The amount of LH released into the medium was assayed by a double antibody RIA with reagents provided by the National Pituitary Agency (Baltimore, MD). The results were expressed in terms of the NIAMDD reference standard rLH-RP-2.

Data points are shown as the means ± standard error in a quadruplicate assay. Statistical differences were analyzed by one-way analysis of variance followed by the Student-Newman-Keuls multiple comparison test.

Results

Effect of GnRH antagonists on GnRH-induced LH release and $[Ca^{2+}]_i$ increase

GnRH antagonists were examined at a fixed dose of 1 μM with increasing doses of GnRH, and their potencies of antagonism were expressed in dose response curves of GnRH-induced LH release (Fig. 1). All antagonists blocked LH release evoked by 10 nM GnRH. The #1-GnRH antagonist, having a reduced LH response to GnRH at 1 nM to 100 nM concentrations, had no effect on LH release by 1 μM GnRH. Its inhibitory effect was weaker than that of other GnRH antagonists. The #2-GnRH antagonist completely inhibited LH responses to GnRH in the concentration range from 100 pM to 1 μM without any inhibitory effect on basal LH release. In another dose response study (Table 1), the LH release induced by 10 nM GnRH was completely inhibited by the same or greater concentrations of the #2-GnRH antagonist. These results indicate that the inhibitory effect of the #2-GnRH antagonist is as potent as that of the #3-GnRH antagonist, the only one used in our previous study.

Cytosolic Ca^{2+} plays a pivotal role in the intracellular mechanism of GnRH action [3, 4]. Table 2 shows the $[Ca^{2+}]_i$ changes induced by each GnRH antagonist at a fixed dose of 1 μM, which is simultaneously applied with 10 nM GnRH. Both the #2- and #3-GnRH antagonists blocked GnRH induced-

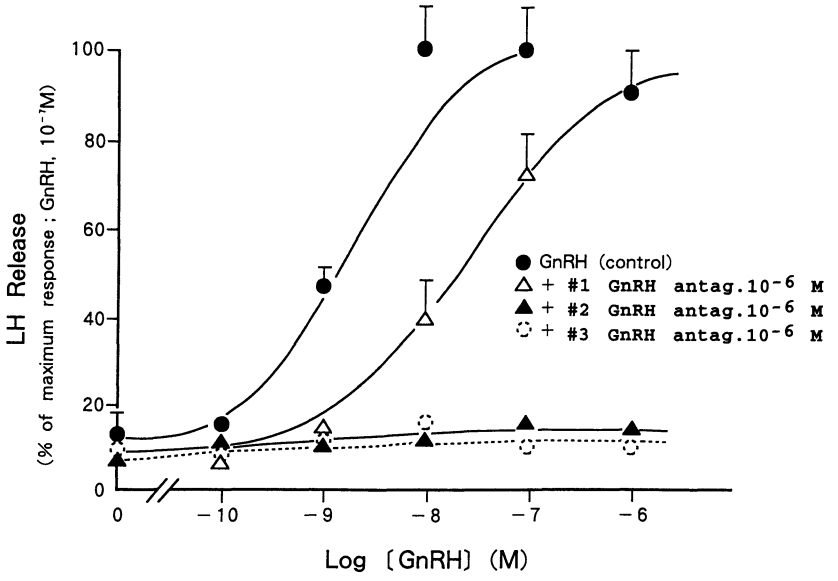

Fig. 1. Inhibitory effects of GnRH antagonists on a dose-response curve of GnRH-induced LH release. The cells were incubated for 4 h with various doses of GnRH alone or in the presence of 10^{-6} M GnRH antagonist. LH in the medium was measured by RIA. Each bar represents the mean ± SEM for quadruplicate cultures.

Table 1. Effect of a #2–GnRH antagonist on GnRH–induced LH release

#2–GnRH antagonist (M)	LH (ng/ml/10^6 cells)	
	control	with GnRH (10^{-8}M)
0	13.4 ± 3.6	118.3 ± 11.3
10^{-10}	9.0 ± 0.7	57.6 ± 2.4
10^{-9}	9.5 ± 0.4	56.5 ± 7.2
10^{-8}	12.8 ± 2.8	16.5 ± 1.6
10^{-7}	11.8 ± 1.9	11.3 ± 1.0
10^{-6}	9.1 ± 0.6	11.5 ± 1.0

The pituitary cells were incubated for 4 h with the indicated doses of the #2- GnRH antagonist in the existence or absence of GnRH. Data are expressed as the mean ± SEM for quadruplicate cultures.

Table 2. Effect of GnRH antagonists on GnRH–induced $[Ca^{2+}]_i$ increase.

GnRH antagonist (M)	$[Ca^{2+}]_i$ (nM)	
	basal	Max after GnRH (10^{-8} M)
... (0 M)	126 ± 3.5	314 ± 9.3
#1 (10^{-6} M)	123 ± 2.8	229 ± 13.6
#2 (10^{-6} M)	111 ± 4.3	114 ± 5.8
#3 (10^{-6} M)	120 ± 3.5	124 ± 3.6

Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was investigated with Fura 2–AM. See the text for a precise description. Each GnRH antagonist was simultaneously applied with 10 nM GnRH. Basal $[Ca^{2+}]_i$ and peak values after the addition are expressed as the mean ± SEM for quadruplicate assays.

$[Ca^{2+}]_i$ increase, while the #1-GnRH antagonist partially inhibited GnRH evoked-$[Ca^{2+}]_i$ increase.

Effect of GnRH antagonists on TPA-induced LH release

Blockade of TPA-induced LH release by GnRH antagonists was investigated with 10 nM TPA and various concentrations of GnRH antagonists. In our previous study, the #3-GnRH antagonist was the only one antagonist which was utilized as an agent to block the interaction between GnRH and its receptor and revealed to have a potent inhibitory effect on TPA-stimulated LH release. However, in the present study, the increasing concentrations of two other GnRH antagonists, #1 and #2, had a slight inhibitory effect on TPA-evoked LH release but was not statistically significant (Fig. 2A).

In another experiment, neither antagonist #1 nor #2 had as clear an inhibitory effect on the dose response curve of TPA-induced LH release as the #3-GnRH antagonist (Fig. 2B).

Discussion

In our previous and present studies, the #3-GnRH antagonist inhibited the action of TPA on LH release. This finding, although reported as minor, was interesting for us, because the post-receptor, intracellular metabolic pathway could be interfered with by blockading the receptor, i.e. the already-evoked downstream event could be blocked by cessation of the upstream event. In the present study, we investigated whether the effect could be applied to all other GnRH antagonists as a general rule.

In the present study, two other GnRH antagonists also were investigated. One of them is a newly synthesized antagonist due for clinical trial in the near future (the #2-GnRH antagonist), and another is a known antagonist in general use for experiments (the #1-GnRH antagonist). Since the #2-GnRH antagonist (10 nM) completely blocked $[Ca^{2+}]_i$ increase and LH release stimulated by 10 nM GnRH, it is as potent as the #3-GnRH antagonist. The inhibiting potency of the #1-GnRH antagonist is weaker than that of other antagonists, proved by both results of the $[Ca^{2+}]_i$ assay and the LH release from pituitary gonadotrophs.

Independently of their potency as GnRH antagonists, the two antagonists, #1 and #2, had no inhibitory effect on TPA-induced LH release contrary to what we expected. This suggests that the inhibitory action on TPA-induced LH release may

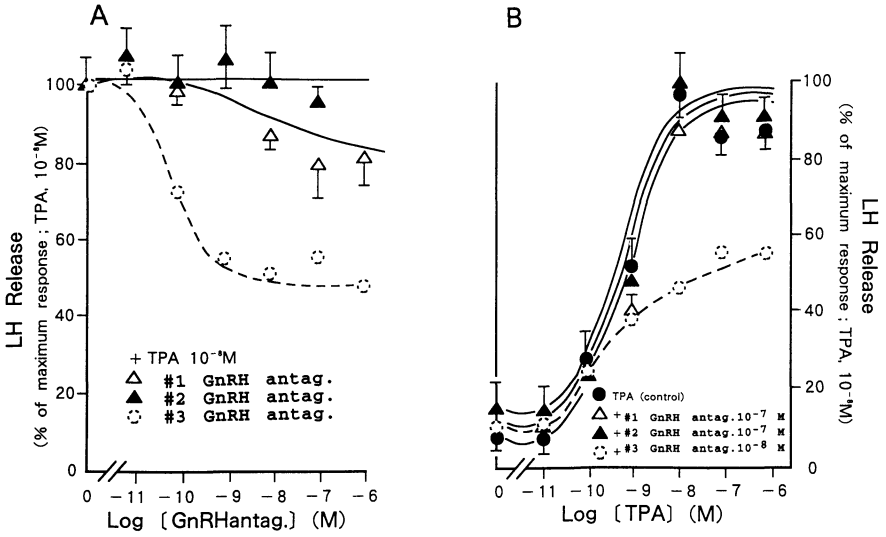

Fig. 2. Dose effect of GnRH antagonists on TPA-induced LH release. Each bar represents the mean ± SEM for quadruplicate cultures. A) The cells were incubated for 4 h wit 10 nM of TPA alone or in the presence of increasing doses of GnRH antagonist. B) The cells were incubated for 4 h with increasing doses of TPA alone or in the presence of a fixed dose of GnRH antagonist.

be a particular action of the #3-GnRH antagonist, and does not support our previous hypothesis that a blockade of GnRH receptor by GnRH antagonist evokes an inhibitory action on C kinase activated-exocytosis. While it is generally accepted that C kinase pathway plays a major role in the GnRH-induced LH release [5, 6], the present study confirmed that not all GnRH antagonists, can inhibit exocytosis through a blockade of the already-activated C kinase system. However, it is still possible that the #3-antagonist may have effect(s) on the TPA, C-kinase or post C-kinase metabolic pathway(s) through the GnRH receptor. Nevertheless, the precise mechanism of the action of the #3-GnRH antagonist remains to be elucidated.

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