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POTENT PITUITARY–GONADAL AXIS SUPPRESSION AND EXTREMELY LOW ANAPHYLACTOID ACTIVITY OF A NEW GONADOTROPIN RELEASING HORMONE (GnRH) RECEPTOR ANTAGONIST "AZALINE B"

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Abstract—We report here the biological characterization of azaline B, a new gonadotropin releasing hormone (GnRH) receptor antagonist, with the following amino acid sequence: [Ac-D-Nal[1], D-Cpa[2], D-Pal[3], Aph[5](atz), D-Aph[6](atz), Ilys[8], D-Ala[10]]-GnRH. Azaline B was shown to suppress several reproductive processes in rats including ovulation, and had very low anaphylactoid activity compared with other GnRH antagonists. Azaline B inhibited histrelin (a GnRH agonist)-mediated follicle stimulating hormone (FSH) and luteinizing hormone (LH) release from cultured rat pituitary cells. Three antagonists ([Nal-Glu]-GnRH, [Nal-Lys]-GnRH ("antide"), and azaline B) inhibited 0.1 nM histrelin-mediated gonadotropin release to baseline levels with EC_{50} values of approximately 0.6 nM. Azaline B, when injected s.c. into rats on the afternoon of proestrus, was more potent at inhibiting ovulation than either [Nal-Glu]-GnRH or [Nal-Lys]-GnRH. The relative order of antiovulatory potencies of the three antagonists was azaline B > [Nal-Glu]-GnRH > [Nal-Lys]-GnRH. Similar azaline B potency was shown by its ability to suppress gonadotropin levels in castrated rats. The improved selectivity of azaline B was demonstrated when it was compared with other GnRH antagonists in the cutaneous anaphylactoid assay (local wheal response) in rats. Results with azaline B were not significantly different from results with vehicle in this assay. [Nal-Glu]-GnRH was more than twice as potent as [Nal-Lys]-GnRH in stimulating a wheal response. Furthermore, the maximal wheal response produced by azaline B was only 0.6 times that of [Nal-Lys]-GnRH, currently one of the most selective antagonists identified. Finally, both azaline B and [Nal-Lys]-GnRH were much less potent than [Nal-Glu]-GnRH in the guinea pig cardiopulmonary anaphylactoid assay after i.v. administration. These data show that azaline B is a potent and selective GnRH receptor antagonist with little or no anaphylactoid activity in animal models, and therefore has potential for use in the treatment of many reproductive endocrine disorders, as well as for use as a contraceptive.

Key words: GnRH receptor antagonist; pituitary; gonadotropin; anaphylaxis; reproduction

Chronic administration of a GnRH‡ receptor agonist results in an inhibition of gonadal function [1–3]. This inhibition of the pituitary–gonadal axis has been employed for the clinical management of several endocrine-related disorders, including precocious puberty, prostate cancer, and endometriosis [4–6]. However, the major disadvantage of agonist treatment is the potential to exacerbate symptoms during the initial stimulatory phase of administration. Since the suppression of gonadal steroid production is the basis of the agonist therapy, it has been clear for many years that a GnRH antagonist would have a distinct advantage over an agonist [7, 8]. This advantage would be due to the immediate inhibitory activity of an antagonist without the potential to exacerbate clinical symptoms.

Many GnRH peptide receptor antagonist analogs have been synthesized, and their potential as therapeutic agents has been demonstrated clearly [8]. However, anaphylactoid reactions such as localized edema, urticaria, cardiopulmonary depression and histamine release were associated with the earlier peptide antagonists, such as [Nal-Arg]-GnRH [9–12]. The next generation of GnRH antagonists with increased potency and improved selectivity was exemplified by [Nal-Glu]-GnRH [13] followed by [Nal-Lys]-GnRH ("antide") [14], which was shown to have low anaphylactoid activity while maintaining antagonist potency [15]. The continued pursuit of improved GnRH antagonists has resulted in the recent synthesis [16, 17] of a novel series of GnRH analogs with N^{ω}-cyanoguanidino moieties ("azalines"). In the present study, we compared the

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‡ Abbreviations: AA, anisole adduct; Ac, acetyl; Aph, 4-amino-phenylalanine; atz, 5′-(3′-amino-1*H*-1′,2′,4′-triazolyl); Cpa, 4-chloro-phenylalanine; FSH, follicle stimulating hormone; GnRH, gonadotropin releasing hormone; D-Glu(AA), D-4-(*p*-[methoxy-benzoyl])-2-aminobutyric acid; Ilys, N^{ε}-isopropyl-lysine; LH, luteinizing hormone; Nal, 3-(2′-naphthyl)-alanine; Nic, nicotinyl; Pal, 3-(3′-pyridyl)-alanine; and RIA, radioimmunoassay.

Table 1. Sequences of GnRH and several agonist and antagonist analogs

GnRH:	pGlu[1]- His[2]-Trp[3]-Ser[4]-Tyr[5]-Gly[6]-Leu[7]-Arg[8]-Pro[9]-Gly[10]-NH_2
Histrelin:	[D-His(*N*-Im-bzl)[6], Pro[9]-NH-C_2H_5]-GnRH
[Nal-Glu]-GnRH:	[Ac-D-Nal[1], D-Cpa[2], D-Pal[3], Arg[5], D-Glu[6](AA), D-Ala[10]]-GnRH
[Nal-Lys]-GnRH:	[Ac-D-Nal[1], D-Cpa[2], D-Pal[3], Nic-Lys[5], D-Nic-Lys[6], Ilys[8], D-Ala[10]]-GnRH
Azaline B:	[Ac-D-Nal[1], D-Cpa[2], D-Pal[3], Aph[5](atz), D-Aph[6](atz), Ilys[8], D-Ala[10]]-GnRH

in vitro and *in vivo* activities of azaline B with those of two other well-characterized GnRH receptor antagonists: [Nal-Glu]-GnRH and [Nal-Lys]-GnRH.

MATERIALS AND METHODS

GnRH analogs

The amino acid sequences of the GnRH analogs used in these studies are shown in Table 1. All GnRH analogs were synthesized at the Salk Institute except for histrelin, which was synthesized by Diosynth (The Netherlands).

Animal assay

All animals were housed at The R.W. Johnson Pharmaceutical Research Institute (PRI) vivarium facility and treated in accordance with the U.S. Laboratory Animal Welfare Act and all of its subsequent revisions and amendments. Room temperature was kept at 25° with a 12 hr light: 12 hr dark cycle (lights on at 6:30 a.m.). Standard food and water were available *ad lib*.

Antiovulatory activity. Female Wistar rats (175–225 g, Charles River, Wilmington, MA) with predictable 4-day estrous cycles were injected s.c. with a GnRH antagonist between 1:00 and 2:00 p.m. on the day of proestrus. The vehicle was 8 mM HCl in normal saline ("acidified saline," pH 3, 299 mOsmol/kg, μOsmette Precision Systems, Natick, MA). In this and all other experiments, solubility was determined by visual inspection, and no cloudiness or precipitation was ever observed. The animals were killed the following morning, and the presence or absence of ova in the oviduct was determined by microscopic inspection. The animals were scored as ovulating if ≥ 1 ovum was present in the oviduct.

Suppression of gonadotropin levels in ovariectomized rats. Female Wistar rats (175–225 g, Charles River) were ovariectomized using Metofane (Pitman-Moore, Mundelein, IL) anesthesia. One week later, one jugular vein was cannulated. The following day, the GnRH antagonists dissolved in acidified saline were injected s.c. Blood samples were then collected through the cannulae. Approximately 350 μL of plasma was obtained from each sampling, and the red blood cells, resuspended in sterile saline, were replaced through the cannula into each rat. The blood collection procedure was followed for studies lasting less than 15 days. For the 15- to 30-day time–course studies, ≤ 1.0 mL blood was collected by heart puncture after Metofane anesthesia. All blood samples were then centrifuged using an IEC HN-SII centrifuge at 1500 rpm for 10 min at room temperature. The resulting plasma was stored at −20° until assayed by RIA for gonadotropin levels.

Anaphylactoid activity. (**A**) *Pulmonary activity.* Male Hartley guinea pigs (440–620 g, Charles River) were anesthetized with urethane (2 g/kg, i.p.; Sigma Chemical Co., St. Louis, MO) and placed in a whole body plethysmograph. A jugular vein and a carotid artery were cannulated for compound administration and monitoring blood pressure, respectively. The trachea was cannulated for respiration at a constant volume using a miniature starling pump (Mart Machine, Raritan, NJ). Transpleural pressure was sensed by a Validyne differential pressure transducer (Buxco Electronics, Sharon, CT) via a 15-gauge needle inserted into the pleural cavity and a sidearm off the tracheal cannula. Tidal volume was sensed by another Validyne differential pressure transducer from pressure changes occurring inside the plethysmograph during each respiration. These signals were input to an online Buxco™ pulmonary mechanics computer (Buxco Electronics) that calculated airway resistance and lung compliance. The guinea pigs were pretreated with succinyl choline (1.2 mg/kg, i.v.) to arrest spontaneous breathing. After a 5-min stabilization period, either acidified saline vehicle (control) or one of the GnRH antagonists was infused i.v. at a total dose of 10 mg in a 1.0 mL vol. over 30 sec. Percent changes from baseline values were recorded at 1, 3, 5, and 30 min after administration. (**B**) *Cutaneous reaction* (local wheal response). Male Wistar rats (500–750 g, Charles River) were injected i.v. with 1.0 mL of 0.5% solution of Evan's blue dye. Various concentrations of GnRH antagonists in acidified saline as well as vehicle control were injected intradermally into a shaved section on the back of the animal. Five injections at separate sites were made into each animal, and were used for determining a dose–response relationship. Fifteen minutes after the intradermal injection, the animals were killed, and the area of each wheal was measured as the product of the longest perpendiculars.

Primary rat pituitary cell culture

Primary rat pituitary cultures were performed as described previously [18]. Briefly, hemisected pituitaries from immature male rats (Wistar, Charles River) were dispersed enzymatically using collagenase followed by viokase. All tissue culture supplies were purchased from GIBCO BRL (Grand Island, NY) unless otherwise specified. Aliquots of a single cell suspension in DMEM/F12 medium supplemented with 8% fetal bovine serum ("FBS,"

HyClone, Logan, UT), 0.52 μg/mL transferrin (Sigma), 0.5 ng/mL parathyroid hormone (Calbiochem, La Jolla, CA), 1 ng/mL basic fibroblast growth factor, 0.29 mg/mL glutamine, 5 μg/mL insulin (Sigma), penicillin/streptomycin and nystatin were plated in a 24-well tissue culture plate and placed in a 5% CO_2 incubator at 37° for 3 days. On day 4, the wells were washed with and reincubated in the nutrient medium. At this time the cultures were treated with 0.1 nM histrelin alone or in combination with various concentrations of the appropriate GnRH antagonist. For the recovery experiments, cultures were treated with a 10 nM concentration of each antagonist alone or in combination with histrelin. After 4 hr at 37°, the media were removed and stored at 4° until assayed for gonadotropin release by RIA as described below.

RIA of gonadotropins

Gonadotropin levels in tissue culture medium or plasma samples were determined by standard RIA techniques using NIDDK kits for rat FSH (RP-2) and rat LH (RP-3).

Statistical analysis

Statistical significance was calculated using Student's *t*-test (for guinea pig pulmonary assay) or by using an analysis of variance program in the SuperANOVA software package (Abacus Concepts, Berkeley, CA). The ED_{50} and $ED_{100\,mm^2}$ values were calculated using either the SAS probit analysis program (SAS Institute, Inc., Cary, NC) or linear regression of log-transformation of response data.

RESULTS

Comparison of [Nal-Glu]-GnRH, [Nal-Lys]-GnRH, and azaline B in rat antiovulatory assay

The activities of the three GnRH antagonists in the rat antiovulatory assay are shown in Fig. 1. The relative order of potency in suppressing ovulation of the three antagonists was azaline B > [Nal-Glu]-GnRH > [Nal-Lys]-GnRH. The calculated ED_{50} (μg/kg) in the rat antiovulatory assay for each of the antagonists studied is given in Table 2. Azaline B was 1.7 and 4.0 times more potent than [Nal-Glu]-GnRH and [Nal-Lys]-GnRH in the rat antiovulatory assay, respectively.

Dose-dependent suppression of plasma gonadotropin levels in ovariectomized rats by azaline B

Azaline B suppressed plasma gonadotropin levels in ovariectomized rats in a dose-dependent fashion. As shown in Fig. 2, a 200 μg/kg injection suppressed FSH within 24 hr and LH within 3 hr. Both FSH and LH returned to normal levels by 3 days at this dose. At the 2000 μg/kg dose, both FSH and LH levels remained suppressed after 8 days. The LH suppression occurred more rapidly than the FSH suppression at both the 200 and 2000 μg/kg doses.

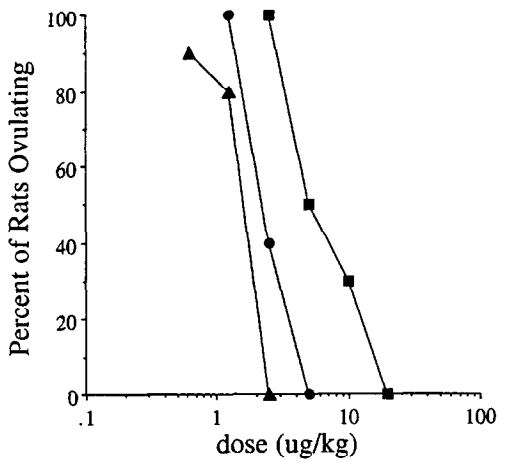

Fig. 1. Effects of [Nal-Glu]-GnRH, [Nal-Lys]-GnRH and azaline B on ovulation in rats. Female rats (N = 10) were injected s.c. between 1:00 and 2:00 p.m. on the day of proestrus with the indicated doses of either [Nal-Glu]-GnRH (●), [Nal-Lys]-GnRH (■), or azaline B (▲). The percent of animals ovulating was determined as described in Materials and Methods.

Comparison of duration of action of [Nal-Glu]-GnRH, [Nal-Lys]-GnRH, and azaline B in the ovariectomized rat model

To compare the duration of action of the three GnRH antagonists, either [Nal-Glu]-GnRH, [Nal-Lys]-GnRH, or azaline B was administered to ovariectomized rats by a single s.c. injection at a dose of 2000 μg/kg. Plasma samples were taken over the following 8 days. The results of this experiment are shown in Fig. 3. [Nal-Lys]-GnRH and azaline B exhibited a significantly longer duration of action than [Nal-Glu]-GnRH. [Nal-Glu]-GnRH suppressed FSH and LH up to 2 days, after which FSH and LH recovered to normal levels by 3–4 days. [Nal-Lys]-

Table 2. Summary of antiovulatory and anaphylactoid activities of [Nal-Glu]-GnRH, [Nal-Lys]-GnRH, and azaline B

	Antiovulatory ED_{50} (μg/kg)	Wheal assay $ED_{100\,mm^2}$ (μg/injection)	Maximum wheal area (mm^2)	Selectivity ratio ($ED_{100\,mm^2}/ED_{50}$)
[Nal-Glu]-GnRH	2.4	0.8	183	0.3
[Nal-Lys]-GnRH	5.6	1.8	14	0.3
Azaline B	1.4	>10	85	>7.1

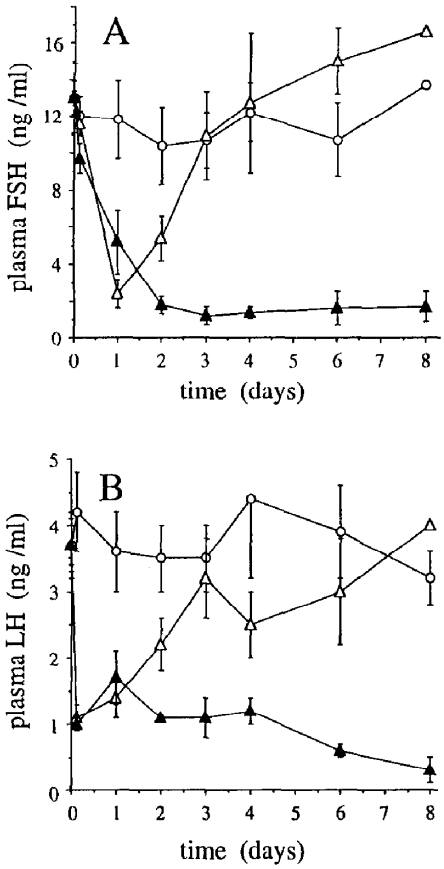

Fig. 2. Effect of two concentrations of azaline B on plasma FSH (A) and LH (B) in ovariectomized female rats. Ovariectomized rats were injected s.c. with vehicle (○), 200 μg/kg (△) or 2000 μg/kg (▲) azaline B at time equals 0 hr. At indicated times after injection, plasma was collected and assayed for FSH and LH as described in Materials and Methods. Each point represents the mean ± SEM (N = 5).

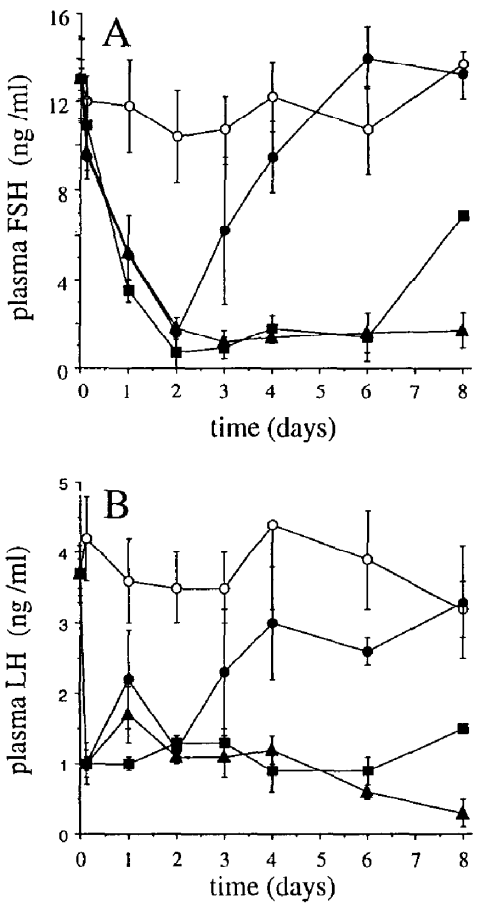

Fig. 3. Effect of 2000 μg/kg of either [Nal-Glu]-GnRH, [Nal-Lys]-GnRH or azaline B on plasma FSH (A) and LH (B) in ovariectomized rats over days 1–8. Ovariectomized rats were injected s.c. with vehicle (○), or 2000 μg/kg of either [Nal-Glu]-GnRH (●), [Nal-Lys]-GnRH (■), or azaline B (▲) at time equals 0 hr. At the indicated time points after injection, plasma was taken and measured for FSH and LH as described in Materials and Methods. Each point represents the mean ± SEM (N = 5).

GnRH and azaline B showed similar duration of action up to 8 days, after which only azaline B continued to suppress levels of both gonadotropins.

To further compare duration of action, either [Nal-Lys]-GnRH or azaline B was administered by a single s.c. injection into ovariectomized rats at 2000 μg/kg, and plasma samples were taken between 8 and 14 days post-injection. Figure 4 shows the results of this experiment. By the end of the 2-week time period, gonadotropin levels were returning to control values in [Nal-Lys]-GnRH-treated rats, whereas the suppression of gonadotropins by azaline B was still evident.

To determine duration of action of azaline B in this model, azaline B was administered by a single s.c. injection into ovariectomized rats at 2000 μg/kg, and plasma samples were taken from 15 to 35 days post-injection. It was not until 25 days for FSH (Fig. 5A) and 30–35 days for LH (Fig. 5B) that the gonadotropin levels in ovariectomized rats treated with azaline B began to approach control values.

We noted that the vehicle control gonadotropin levels in both Figs. 4 and 5 were lower than in the non-cannulated zero-time point controls. This may be due to stress associated with cannulation since chronic stress has been shown to affect gonadotropin levels in many species, including the rat [19, 20].

Comparison of [Nal-Glu]-GnRH, [Nal-Lys]-GnRH, and azaline B in rat primary pituitary cell cultures

The relative potencies of the three GnRH antagonists were compared in cultures of isolated pituitary cells in order to study the direct action of these compounds at the level of the gonadotrope. As shown in Fig. 6, [Nal-Glu]-GnRH, [Nal-Lys]-GnRH and azaline B appeared to be equipotent in suppressing the histrelin-mediated release of both FSH and LH. The EC_{50} (the concentration of antagonist resulting in a 50% reduction of histrelin-mediated gonadotropin release) of all three antag-

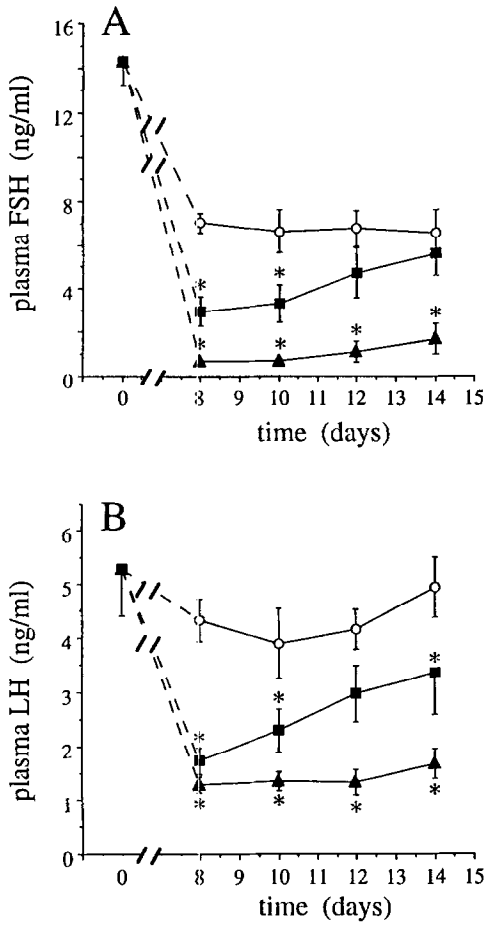

Fig. 4. Effect of 2000 μg/kg of either [Nal-Lys]-GnRH or azaline B on plasma FSH (A) and LH (B) in ovariectomized rats 8–14 days post-injection. Ovariectomized rats were injected s.c. with vehicle (○), or 2000 μg/kg of either [Nal-Lys]-GnRH (■) or azaline B (▲) at time equals 0 hr. At the indicated time points, plasma was taken and measured for FSH and LH as described in Materials and Methods. Key: (*) $P < 0.05$ vs control. Each point represents the mean ± SEM (N = 6).

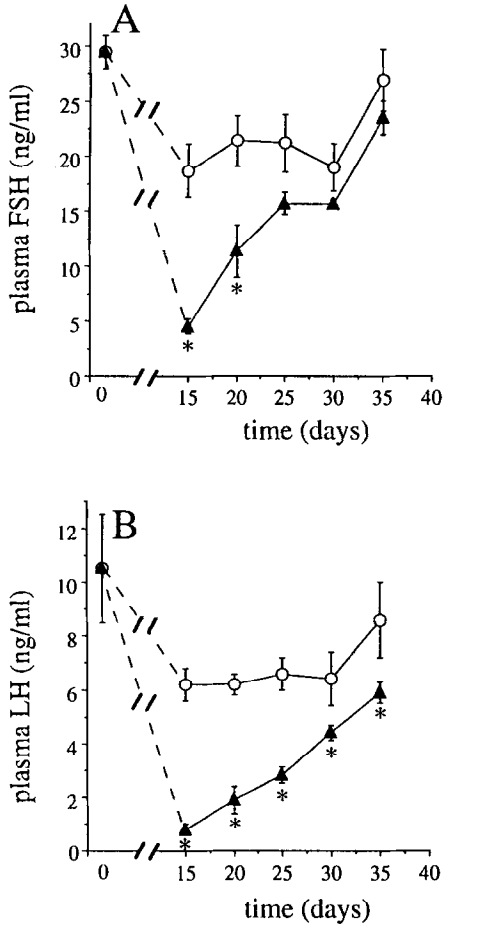

Fig. 5. Effect of 2000 μg/kg of azaline B on plasma FSH (A) and LH (B) in ovariectomized rats 15–35 days post-injection. Ovariectomized rats were injected s.c. with vehicle (○), or 2000 μg/kg of azaline B (▲) at time equals 0 hr. At the indicated time points, plasma was taken and measured for FSH and LH as described in Materials and Methods. Key: (*) $P < 0.05$ vs control. Each point represents the mean ± SEM (N = 6).

onists was approximately 0.6 nM. To assess the potential contribution of toxicity of the antagonists to the pituitary cells, cultures were treated with a single high concentration (10 nM) of each of the antagonists and then co-incubated with increasing concentrations of histrelin for 4 hr. As shown in Fig. 7, increasing concentrations of histrelin completely reversed the inhibition caused by either [Nal-Glu]-GnRH, [Nal-Lys]-GnRH, or azaline B.

Comparison of [Nal-Glu]-GnRH, [Nal-Lys]-GnRH, and azaline B in the guinea pig pulmonary anaphylactoid assay

As shown in Fig. 8, neither azaline B (10 mg, i.v.) nor [Nal-Lys]-GnRH (10 mg, i.v.) demonstrated any significant anaphylactoid activity as measured by either increased airway resistance (A) or decreased lung compliance (B) in the guinea pig model. In contrast, [Nal-Glu]-GnRH (10 mg, i.v.) showed significant anaphylactoid activity, producing over a 1000% increase in airway resistance and an 80% decrease in lung compliance.

Comparison of [Nal-Glu]-GnRH, [Nal-Lys]-GnRH, and azaline B in the rat cutaneous anaphylactoid assay

To characterize further the anaphylactoid activity of the three GnRH antagonists, [Nal-Glu]-GnRH, [Nal-Lys]-GnRH, and azaline B were evaluated for localized wheal activity in the rat. Both [Nal-Glu]-GnRH and [Nal-Lys]-GnRH demonstrated significant activity in this assay as shown in Fig. 9, with [Nal-Glu]-GnRH more than twice as potent as [Nal-Lys]-GnRH. Azaline B, however, did not significantly stimulate a wheal even at the highest dose tested (10 μg) compared with the vehicle control. The $ED_{100\,mm^2}$ for each antagonist (the

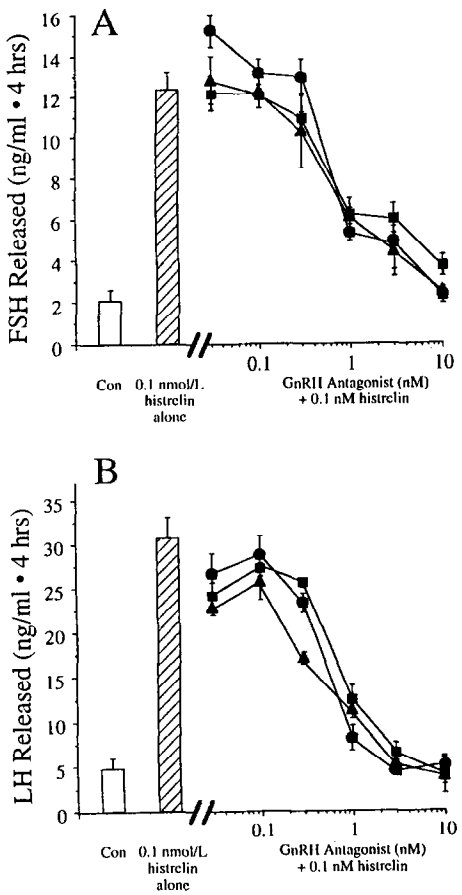

Fig. 6. Effects of [Nal-Glu]-GnRH, [Nal-Lys]-GnRH and azaline B on histrelin-mediated release of FSH (A) and LH (B) from rat pituitary cell cultures. Cultures of rat pituitary cells were treated with 0.1 nM histrelin in the absence or presence of increasing concentrations of either [Nal-Glu]-GnRH (●), [Nal-Lys]-GnRH (■), or azaline B (▲) for 4 hr at 37°. Media were then removed and assayed for FSH and LH by RIA as described in Materials and Methods. Control (con) represents the value of basal release of gonadotropins from untreated cultures. Each point represents the mean ± SEM (N = 3).

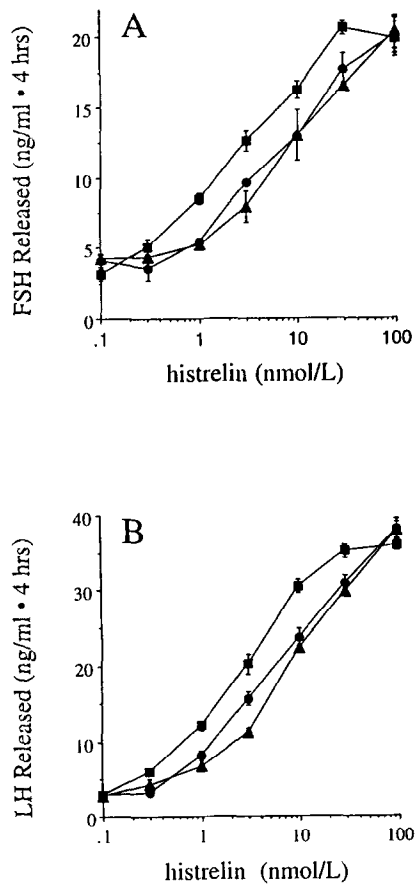

Fig. 7. Recovery of FSH (A) and LH (B) release in the presence of 10 nM [Nal-Glu]-GnRH, [Nal-Lys]-GnRH or azaline B with increasing concentrations of histrelin. Cultures of rat pituitary cells were treated with a 10 nM concentration of either [Nal-Glu]-GnRH (●), [Nal-Lys]-GnRH (■), or azaline B (▲) in the presence of increasing concentrations of histrelin for 4 hr at 37°. Media were then removed and assayed for FSH and LH by RIA as described in Materials and Methods. Each point represents the mean ± SEM (N = 3).

microgram dose that produced a 100 mm^2 wheal) and the maximal wheal size produced (area in mm^2) are shown in Table 2. The relative potency of azaline B could not be calculated in this assay since none of the doses of azaline B tested caused a wheal response that was significantly different from control. Therefore, the $ED_{100\,mm^2}$ for azaline B in this assay can only be estimated as greater than the highest dose tested (> 10 μg). This low anaphylactoid activity of azaline B was demonstrated further by comparison of the maximal wheal area produced. The maximal wheal areas produced by [Nal-Glu]-GnRH, [Nal-Lys]-GnRH, and azaline B were 183, 134 and 84 mm^2, respectively (Table 2).

Selectivity ratio of [Nal-Glu]-GnRH, [Nal-Lys]-GnRH, and azaline B

The relative selectivities (the degree to which a compound produces the desired effect in relation to the adverse effect) of [Nal-Glu]-GnRH, [Nal-Lys]-GnRH, and azaline B were estimated by calculating the ratio of the $ED_{100\,mm^2}$ as determined in the rat cutaneous anaphylactoid assay to the ED_{50} as determined in the rat antiovulatory assay. Table 2 shows that azaline B possessed the highest selectivity ratio when compared with those of the other two antagonists. The selectivity ratio of azaline B was >7.1, whereas the selectivity ratio of both [Nal-Lys]-GnRH and [Nal-Glu]-GnRH was 0.3.

DISCUSSION

The identification of GnRH receptor antagonists began within 1 year of the elucidation of the structure of porcine GnRH [21]. However, the therapeutic potential for such agents was questioned when anaphylactic reactions were seen after administration

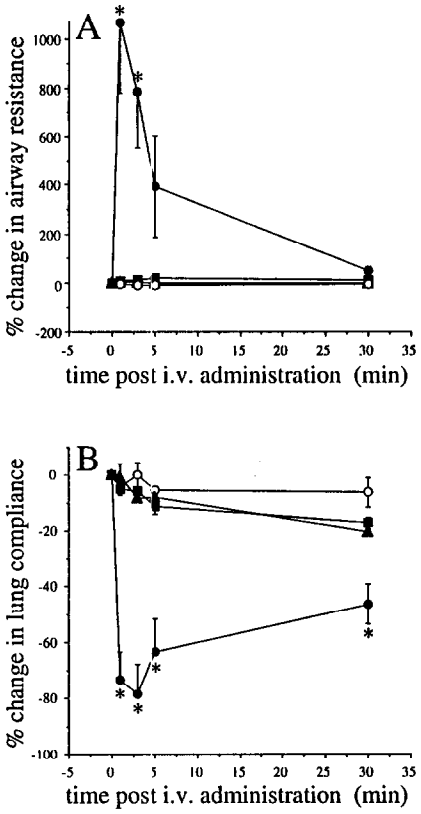

Fig. 8. Effects of [Nal-Glu]-GnRH, [Nal-Lys]-GnRH and azaline B on pulmonary activity in the guinea pig. At time equals 0 min, either vehicle (○), or 10 mg of [Nal-Glu]-GnRH (●), [Nal-Lys]-GnRH (■), or azaline B (▲) was administered as described in Materials and Methods. Pulmonary function measured as airway resistance (A) and lung compliance (B) was calculated using an online Buxco™ pulmonary mechanics computer at 1, 3, 5, and 30 min. Key: (*) $P < 0.05$ vs control. Each point represents the mean ± SEM ($N = 3$ animals for [Nal-Lys]-GnRH and azaline B, $N = 6$ for [Nal-Glu]-GnRH).

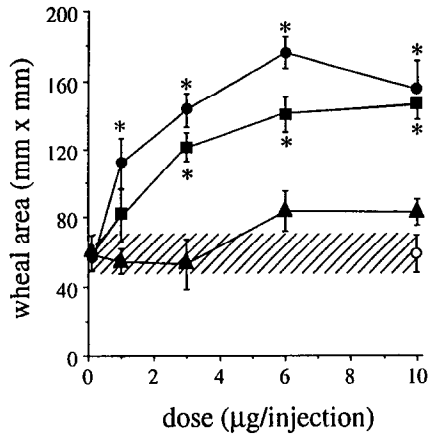

Fig. 9. Effects of [Nal-Glu]-GnRH, [Nal-Lys]-GnRH and azaline B on a localized wheal response in rats. At time equals 0 min, vehicle (○), or 1, 3, 6, or 10 μg of either [Nal-Glu]-GnRH (●), [Nal-Lys]-GnRH (■), or azaline B (▲) was injected intradermally into rats preinjected i.v. with 1.0 mL of Evan's blue dye. After 15 min, the area of the wheals produced was calculated as described in Materials and Methods. Key: (*) $P < 0.05$ vs control. Each point represents the mean ± SEM ($N = 7$). Hash marks indicate mean control and SEM.

of the earliest antagonists. Since that time, research has been directed toward finding more potent and less anaphylactic GnRH antagonists for potential clinical use. In the current study, we have characterized the activity of one such antagonist, azaline B, in several animal models. These studies show that azaline B is a novel GnRH antagonist with increased potency and lower anaphylactoid activity compared with [Nal-Glu]-GnRH and [Nal-Lys]-GnRH, the latter considered to be very selective.

The primary site of action of azaline B is probably at the pituitary level, since azaline B was shown to have a high affinity to isolated pituitary membranes [16]. This finding suggests a direct binding of this peptide to GnRH receptors and therefore implies a competitive mechanism of action. The *in vitro* pituitary cell culture experiments in the current study in which the inhibition of gonadotropin release mediated by azaline B was reversed with increasing concentrations of the GnRH agonist histrelin add further evidence for a competitive mechanism of action of azaline B. Although there is convincing evidence that GnRH receptors also occur in rat gonadal tissues [22], it is unknown if an interaction between azaline B and these receptors would alter the observed biological activity of azaline B.

The low ED_{50} of 1.4 μg/kg for azaline B in the rat antiovulatory assay demonstrates its greater *in vivo* potency compared with [Nal-Glu]-GnRH and [Nal-Lys]-GnRH in this assay. The basis of this greater potency *in vivo* as opposed to the equipotency of all three antagonists at inhibiting the release of immunoreactive gonadotropins from pituitary cell cultures *in vitro* may be due to an alteration in the pharmacodynamics or pharmacokinetics of the compounds *in vivo*. For example, [Nal-Lys]-GnRH (antide) has been shown to have significant binding to serum proteins [23], which has been used to help explain its long duration of action. Since an extended duration of action may, in general, translate into an observed increase in potency, it may be that azaline B binds to similar serum proteins or has a difference in tissue uptake resulting in an increase in its serum half-life. Such studies would clearly be of interest. Another potential explanation of the potency differences *in vivo* versus *in vitro* may be the result of the difference between immunoreactive and bioactive gonadotropins. The pituitary cell culture assays used in this study measured gonadotropin release by immunoreactivity, whereas ovulation is a result of biologically active gonadotropins. Potency differences in the *in vitro* pituitary cell culture assay may have been revealed if gonadotropin release was measured by a bioassay and not an immunoassay.

Indeed, recent findings* suggest that azaline B is more potent at suppressing release of bioactive gonadotropins from pituitary cell cultures *in vitro* than either [Nal-Glu]-GnRH or [Nal-Lys]-GnRH. Future studies will determine if this effect of azaline B on the release of bioactive gonadotropins *in vitro* explains its greater relative potency *in vivo*.

Azaline B showed negligible anaphylactoid activity in two types of anaphylactoid assays—one reflecting a localized response and the other a more systemic response. In the systemic assay which examined pulmonary function in the guinea pig, both azaline B and [Nal-Lys]-GnRH were devoid of activity. However, in the localized wheal response assay in the rat, only azaline B was devoid of activity even at the high dose tested (10 μg/injection). The positive anaphylactoid response of [Nal-Lys]-GnRH seen only in the wheal assay but not in the pulmonary assay is similar to that reported earlier [24] and may be due to a direct effect on local mast cell release of mediators of anaphylaxis, but not in such quantities as to produce the more systemic, i.e. bronchospastic, response. The reduced activity of azaline B in both of these assays suggests that azaline B has minimal potential for anaphylactoid activity. In direct measurements of histamine release from mast cells *in vitro*, azaline B has shown extremely low activity [25], consistent with our findings. The minimal anaphylactoid activity and potent antiovulatory activity of azaline B are reflected in its favorable selectivity ratio.

A single injection of azaline B exhibited a significantly longer duration of action than either [Nal-Glu]-GnRH or [Nal-Lys]-GnRH in the ovariectomized female rat model measuring the suppression of gonadotropins. The duration of action of azaline B was dose dependent in these studies. The difference in the duration of action between azaline B, [Nal-Glu]-GnRH and [Nal-Lys]-GnRH in the whole animal is not likely to be due to differences at the pituitary level, since the GnRH agonist histrelin was approximately equipotent at reversing the action of all three GnRH antagonists in the *in vitro* cell culture system. The cause of the long duration of action of azaline B is unknown and currently under investigation.

In summary, the findings in these studies indicate that azaline B is a potent GnRH receptor antagonist with extremely low anaphylactoid activity in animal models and, as such, has the potential to be used as a therapeutic agent for clinical situations requiring inhibition of pituitary gonadotropin secretion.

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