Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 5759–5763, July 1992
Medical Sciences

Evaluation of luteinizing hormone-releasing hormone antagonistic activity *in vitro*

(luteinizing hormone-releasing hormone analogues/*in vitro* bioassay/superfusion/luteinizing hormone-releasing hormone receptor interactions)

VALÉR J. CSERNUS* AND ANDREW V. SCHALLY

Endocrine Polypeptide and Cancer Institute, Veterans Administration Medical Center, and Department of Medicine, Tulane University School of Medicine, New Orleans, LA 701460

Contributed by Andrew V. Schally, March 16, 1992

ABSTRACT **Antagonistic analogues of luteinizing hormone-releasing hormone (LHRH) belong to a class of compounds that can be utilized for treatment of some hormone-dependent cancers and gynecologic disorders. Recently, we synthesized and tested a large number of LHRH analogues for LHRH antagonistic activity in the dispersed pituitary cell superfusion system. This fast, reliable, and dynamic system made it possible for us not only to evaluate the relative amounts of an analogue required for suppression of the LH-releasing activity of exogenous LHRH but also provided quantitative data on dynamic interactions between the LHRH analogue, LHRH receptors, and LH secretion. Three experimental paradigms were used: (*i*) LHRH responses after preincubation with the antagonist, (*ii*) pulsatile, simultaneous infusion of LHRH and the antagonistic analogue, and (*iii*) effects of the analogues on ongoing, continuous LH secretion induced by prolonged stimulation with LHRH. From the data obtained, we conclude that (*i*) the suppression of the LHRH-induced LH release was more effective and longer lasting when the cells were preincubated with the antagonistic analogues before the LHRH stimulation than in the case of simultaneous exposure; (*ii*) not only the potency but also the time of onset and the duration of the LH release-suppressing activity varied according to the different peptides used, resulting in different shapes of response curves; and (*iii*) from the accurate data obtained in this dynamic system, quantitative parameters of the *in vivo* interactions between the antagonists and LHRH on the LHRH receptor can be calculated.**

In the past few years, a large number of antagonists of luteinizing hormone-releasing hormone (LHRH) has been synthesized and evaluated for therapeutic use (1–6). These analogues may have various practical applications, including treatment of gynecologic disorders and hormone-sensitive tumors such as prostate and breast cancers (1, 2, 7–9). Although chronic administration of LHRH agonists is necessary for inhibition of LH, a single dose of a potent LHRH antagonist is sufficient to evoke the same effect (1, 2, 5–13). Agonists, unlike antagonists, induce a LH surge before the LH secretion blockade takes effect, which may result in an undesirable flare-up (1, 2). The bioactivity of a newly synthesized peptide must be tested by the most efficacious means. Although the final evaluation always has to be made *in vivo*, for screening purposes the simpler, less expensive, faster, and more accurate *in vitro* bioassays are the methods of choice. *In vitro* assays are especially suitable to test hypothalamic hormones since (*i*) their target organ, the pituitary, can be obtained easily; (*ii*) the pituitary cells survive and function well in an artificial environment; and (*iii*) RIA methods are widely available to measure the response, based on the pituitary hormone release.

In vitro pituitary bioassays were described as early as 1955 (14). Although this static system provided valuable help in isolation and characterization of several hypothalamic hormones (15), this method is unsatisfactory for providing data on the dynamics of hormone release. The static system may also be less informative when more complex approaches, such as determining the bioactivity of antagonistic analogues, are used. During the several hours of incubation, the released hormones and other metabolic products accumulate in the medium and may affect the metabolism of the cells and reduce further hormone secretion. Proteolytic enzymes, present in the incubation medium, may also digest the test material and the secreted hormone.

Systems in which tissue culture medium is perfused continuously through surviving tissues are devoid of these drawbacks. Thus, it is possible to add the test material in a more physiological, pulsatile way (16). The sampling can also be more refined and, consequently, the dynamics, the timing, and subtle changes in the responsiveness can also be analyzed. Although the terminology has not been standardized, the method is generally called "perifusion" when whole organs or organ segments are used and "superfusion" when dispersed cells are utilized (16).

Recently we designed, synthesized, and tested >100 antagonistic LHRH analogues (1, 2, 5, 6, 11). Some of them proved to be highly potent and free from edematogenic effects. The LHRH antagonists were also very effective in suppressing the growth of experimental mammary cancers and prostate tumors (7, 9). To assess the bioactivity of these compounds, the dispersed rat pituitary superfusion system was initially used (5, 6). Utilizing this dynamic method, various experimental techniques were used in search of the most informative approach. In the course of our work, we also wanted to obtain data on the reliability of this *in vitro* system in testing antagonistic analogues of LHRH and acquire a deeper insight into the physiology of pituitary hormone secretion. This paper summarizes and analyses our experiences with the *in vitro* superfusion system related to control of LH secretion from pituitary cells.

MATERIALS AND METHODS

Superfusion. Dispersed rat pituitary cell superfusion was performed as described (16). Briefly, anterior pituitaries of young adult female Sprague–Dawley rats were digested with

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Abbreviations: LH, luteinizing hormone; LHRH, LH-releasing hormone; NET INT, net integral; Nal(2), 3-(2-naphthyl)alanine; Pal(3), 3-(3-pyridyl)alanine; Cit, citrulline; Hci, homocitrulline; Phe(4Cl), 4-chlorophenylalanine; Moc, methyloxycarbonyl.

*Present address: Department of Anatomy, University Medical School Pécs, H-7643 Pécs, Hungary.

collagenase (no. CLS-II; Worthington) for 1 hr followed by a mechanical dispersion. The resulting cell suspension from 1.5 pituitaries, containing mostly small clusters of cells, was then sedimented together with a suspension of Sephadex G-10 (Sigma) and packed into 6.6-mm columns. The dead volume of the system was carefully set to 1 ml. Tissue culture medium 199 (Sigma) with supplements (16), equilibrated with 95% air/5% carbon dioxide, was perfused through the columns at a flow rate of 0.33 ml/min. After an overnight recovery period, during which the baseline stabilized and the cells regained their full responsiveness (16), the samples to be tested were introduced through a four-way valve. During the 8.5- to 9-hr experimental period, 170–180 one-ml fractions were collected. The system was standardized with 3-min exposures to 100 mM potassium chloride (16) or 3 nM LHRH. At the end of each experiment, the total immunologically detectable LH content of the cells was determined from 0.01 M hydrochloric acid extracts. The samples were introduced in various concentrations, generally for 3–9 min (time of 1–3 fractions), at 30-min intervals. Detailed parameters of the large variety of sample administration schemes are described with the results for clarity.

RIA. Rat LH levels were measured from aliquots (50 μl) of the collected medium effluent using kits from the National Institute of Diabetes and Digestive and Kidney Diseases (National Institutes of Health). As a standard, rat LH-RP2 reference preparation was used. The sensitivity limit of the assay was 0.12 ng/ml.

Data Analysis. Quantitative evaluation of the superfusion results was performed with a state of the art computer program (16). Further evaluations were based on the net integral (NET INT) and T50 (biological half-time of the response) values. The variances were negligible. Data on the graphs were based on values obtained from 3 to 10 experiments. The results were standardized with respect to LH response following the first exposure to potassium. The types and the doses of the test materials were placed in statistically counterbalanced sequence in the repeated experiments unless otherwise indicated. In the response plots, the signs for the sample administration were corrected with the dead volume of the system. As a consequence of this, the time lags between the sample administration (marked as filled bars on the figures) and the responses represent only the delays in the responses of the cells.

Peptides. LHRH analogues [Ac-D-Nal(2)^1,D-Phe(4Cl)^2,D-Trp^3,D-Hci^6,D-Ala^{10}]LHRH (SB-29), [Ac-D-Nal(2)^1,D-Phe(4Cl)^2,D-Pal(3)^3,D-Cit^6,D-Ala^{10}]LHRH (SB-75), [Ac-D-Nal(2)^1,D-Phe(4Cl)^2,D-Pal(3)^3,Arg^5,D-Lys^6,D-Ala^{10}]LHRH (SB-92), and [Moc-D-Phe^1,D-Phe(4Cl)^2,D-Trp^3,D-Cit^6,D-Ala^{10}]LHRH (SB-102) were designed, synthesized, and purified in our laboratory as described (5, 6). [Nal(2), 3-(2-naphthyl)alanine; Pal(3), 3-(3-pyridyl)alanine; Cit, citrulline; Hci, homocitrulline; Phe(4Cl), 4-chlorophenylalanine; Moc, methyloxycarbonyl.]

RESULTS

LH Response to LHRH Stimulation. Stimulation of the dispersed rat pituitary cells for 3 min with 3 nM LHRH resulted in a sharp, transient increase in LH secretion. The onset of the LH release was very rapid: the leading edge of the response peak followed the beginning of the stimulus within 20 sec, as revealed by data from experiments with a shorter fraction time. The LH release also stopped rapidly. The elevated LH level returned to the baseline with 6.66 ± 0.42 min half-time (T50). Cells of one pituitary secreted 26 ± 2.15 ng of LH during a response (NET INT value). When the stimulation was repeated at 30-min intervals for 8 hr, distinct peaks of similar size and shape were obtained (Fig. 1). The area of the peaks (NET INT) increased gradually during the first 90 min up to 127% and subsequently decreased slowly, final response being 68% of the first response. These changes of the responsiveness were consistent throughout the experiments. All data obtained during tests with antagonists, which included repeated LHRH exposures, were corrected in accordance with these results.

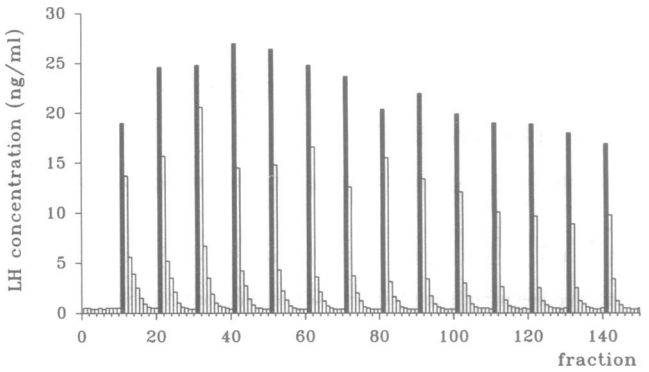

FIG. 1. LH release from dispersed rat pituitary cells in response to repeated, pulsatile stimulation with 3 nM LHRH for 3 min at 30-min intervals.

Prolonged exposure of the cells to 1 nM LHRH induced a long, continuous increase in LH secretion (Fig. 2, response C). The elevated LH concentration in the collected samples further increased during the first 90 min and then gradually declined later, but the LH concentration, even at the end of the 150-min period, did not fall below that of the first fraction. These data revealed no significant signs of desensitization in our system during this period. The changes in the LH secretion showed close correlation with results obtained following repeated, pulsed stimulation.

LH Response to Antagonists. Powerful LHRH antagonist SB-75 was given at 30 nM concentration for 12 min. Although the basal LH secretion of the cells (0.55 ± 0.08 ng/ml) was well above the sensitivity limit of the RIA (0.12 ng/ml), no significant decrease in LH secretion was detected, provided sufficient time had elapsed after a nonspecific or specific stimulation to obtain the "real" basal secretion (Fig. 3, response B). Similarly, no changes of the basal secretion were detected during the preincubation period with the antagonists described in the next series of experiments (Fig. 3, response G, and Fig. 5, response F).

However, an effective, long-lasting blockade of the specific LH-releasing mechanism is indicated by the marked depression of the LH responses to subsequent stimulation

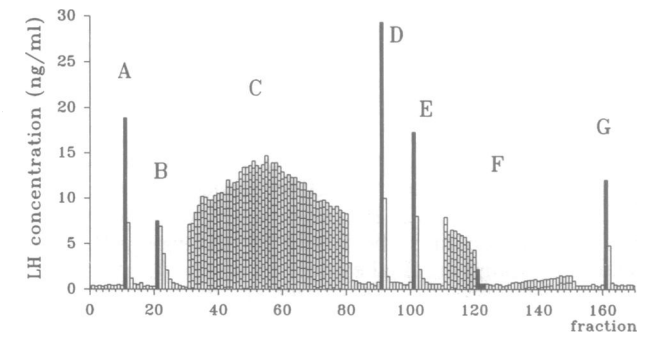

FIG. 2. Effect of LHRH antagonist SB-75 on ongoing LH release from dispersed rat pituitary cells. The cells were stimulated with continuous exposure to 1 nM LHRH for 90 min (C). Using a similar design, SB-75 was introduced 30 min after the onset of the LHRH stimulus at 30 nM concentration for 9 min (F). The responsiveness of the system was standardized by analyzing the LH responses to 3-min exposures with 3 nM LHRH (B and E) or 100 mM potassium chloride (A, D, and G).

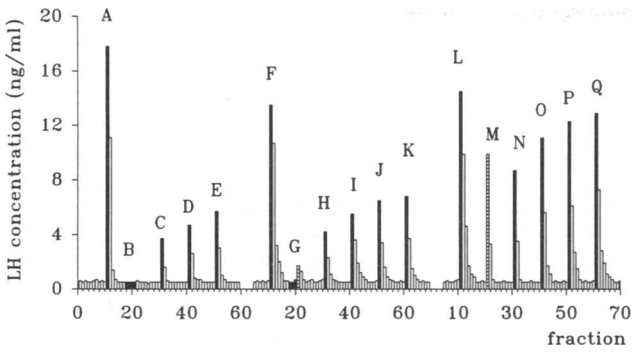

FIG. 3. Changes in LH responsiveness of rat pituitary cells exposed to a powerful LHRH antagonist, SB-75, *in vitro*. The cells were stimulated with 3 nM LHRH for 3 min before (A, F, and L) or after (C–E, H–K, and N–Q) the antagonist was introduced. SB-75 was given at 30 nM concentration for 9 min alone (B), at 3 nM concentrations for 9 min and then together with 3 nM LH-RH for 3 min (G), or for 3 min in a mixture of 30 nM SB-75 and 3 nM LHRH (M).

with LHRH (Fig. 3, responses C–E). In cases in which a detectable decrease in the "basal" LH secretion was experienced following administration of an analogue, the cells proved to be in a slightly stimulated condition because of (*i*) too short a time had elapsed after a test stimulation, (*ii*) the recovery period after the column preparation (mechanical stimulation) was too short (<5 hr), or (*iii*) the cells had not recovered from a temporary mechanical or chemical stress due to malfunctions of the system (vibration of the column, air bubble entering or forming in the column, or the pressure of the medium had changed as a consequence of a partial block of the medium flow).

LH Response After Preincubation with Antagonists. In these experiments, the cells were exposed to a 3 nM solution of the antagonists for 9 min and then to a mixture of 3 nM concentration of the same antagonist and 3 nM LHRH for an additional 3 min (Fig. 3, response G). To test changes in LH responsiveness of the cells, 3 nM LHRH was also given for 3 min at 30-min intervals, three or four times thereafter (Fig. 3, responses H–K).

LH response to LHRH was markedly reduced after exposure of the cells to the antagonist, followed by a gradual recovery period. Using this experimental protocol, different patterns in the alterations of the LH responsiveness to LHRH were found following the exposure to antagonistic LHRH analogues, depending on their chemical structure. Fig. 4 shows examples for the four fundamental LH release patterns

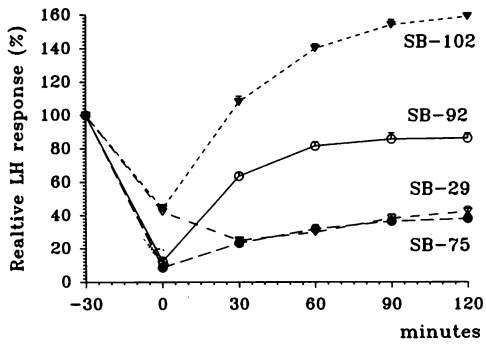

FIG. 4. Patterns of changes in LH responsiveness of rat pituitary LH cells *in vitro* following 12-min exposures to various LHRH antagonists: SB-92, SB-75, SB-29, and SB-102. The cells were stimulated with 3 nM LHRH during the last 3 min of the exposure to antagonist and four times thereafter for 3 min at 30-min intervals. NET INT values of the response curves are plotted against elapsed times.

we obtained. These were (*i*) fast appearance followed by a rapid disappearance of the inhibition of LH release (SB-92), (*ii*) fast onset of the release blockade with a long-lasting inhibitory effect (SB-75), (*iii*) gradual increasing effect even after a single, short exposure (SB-29), and (*iv*) significant augmentation of the LH release, following a brief inhibitory period (SB-102). Some of our antagonists (e.g., SB-75, SB-29) proved to be very potent, for instance, following a single exposure at 3 nM concentration, and the normal LH responsiveness was not reestablished even by the end of the 8-hr experiment, rendering the system unfit for further analysis.

Regression analysis was performed on the relative inhibitory effect of the analogues (% of the NET INT values of the response curve) plotted against time. From these data, the time required for complete recovery of the LH responsiveness can be calculated. Based on these calculations the recovery times for SB-92, SB-75, SB-29, and SB-102 were 3.8 hr, 35.8 hr, 46.5 hr, and 10.9 min, respectively.

Testing of LHRH Antagonist with Simultaneous Administration of LHRH and the Antagonist. A mixture of 30 nM antagonist and 3 nM LHRH was applied for 3 min (Fig. 3, response M), followed by four consecutive LHRH exposures (3 nM for 3 min) at 30-min intervals (Fig. 3, responses N–Q). Although the concentration of the peptides was 10 times higher than that in the previous series of experiments, the inhibition of the LH release was greatly diminished, both in intensity and duration (compare Fig. 3, responses G–K with M–Q).

Effects of Antagonists on Ongoing LHRH-Induced LH Release. In this series of experiments, the cells were exposed to 1 nM LHRH continuously for 150 min. Thirty minutes after the start of LHRH stimulation, 30 nM antagonist SB-75 was also infused for 3 min (Fig. 2, response F). The analogue caused an immediate reduction (within 1 min) in LH secretion, which showed a tendency for a recovery, but did not reach control levels before the end of the LHRH infusion.

Interaction Between the Antagonist Analogue and the Potassium Response. Temporary increase in K^+ concentration in the medium is a frequently used, nonspecific, but reproducible way of stimulating of the peptide secretion. In our system, 100 mM increase in K^+ concentration for 3 min results in a rapid, brief LH response, similar in area (NET INT value) to that obtained after 3 nM LHRH stimulation (16) (Fig. 5, responses A, B, and E).

To check if the potassium response is modified by the antagonist, previous experiments were repeated by substi-

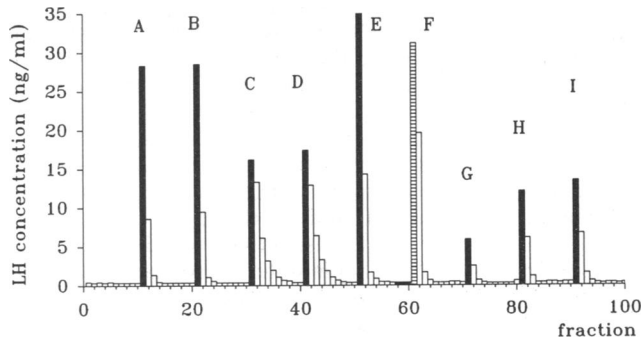

FIG. 5. LH responses to temporary elevation of potassium concentration in dispersed rat pituitary superfusion. Potassium concentration of the medium was elevated by 100 mM for 3 min (A, B, E, and F). The increase in LH secretion is transitory and the subsequent responses to LHRH (3 nM for 3 min) (C and D) are not altered. A 9-min preincubation with 3 nM SB-75 followed by simultaneous exposure to 3 nM SB-75 with 100 mM potassium chloride did not decrease the response to potassium significantly (F). However, an effective blockade of the release mechanism is indicated by the diminished LH responsiveness to consecutive LHRH stimuli (G–I).

tuting one of the exposures to LHRH with 100 mM potassium. One of our most potent LHRH antagonists, SB-75, even after a 9-min preincubation at a dose of 3 nM, did not affect significantly the LH response to potassium (Fig. 5, response F).

DISCUSSION

In vitro characterization of the responses to LHRH antagonistic analogues greatly facilitates the evaluation of the bioactivity of the newly synthesized compounds. The dispersed cell pituitary superfusion system proved to be suitable to analyze not only the relative potency of the antagonists but also the dynamics of the action of the analogues on pituitary LH cells.

Based on the data obtained, we can draw the following conclusions.

(*i*) The pituitary cells in our superfusion system respond to pulsatile and continuous LHRH stimulation in a predictable, reliable way. Slight changes in the responsiveness, which were consistent throughout several experiments, might be a consequence of the combined effects of (*a*) the increase in LH synthesis, (*b*) depletion of the intracellular LH reserves, and (*c*) changes in the state of the release mechanism.

(*ii*) Continuous stimulation with 1 nM LHRH for 150 min does not induce significant desensitization in our system. The responsiveness under this condition changes in a manner similar to that following pulsatile stimulus with 3 nM LHRH. Several authors claimed that LH responsiveness decreased much more rapidly following continuous LHRH stimulation than after pulsatile stimulation (17–20). Analyses of the data presented in these papers reveal that in those experiments, the integral dose of LHRH used for stimulation and the total amounts of the released LH were much higher during continuous stimulation than during the control, pulsatile stimulation. Consequently, a significantly faster decrease of the intracellular LH reserves and/or faster exhaustion of the specific release mechanisms occurred, rendering impossible any comparison of the results of the two experimental groups. In our experiments, the average rates of LH release from the cells were similar following 1 nM continuous stimulation or after exposure to 3 nM LHRH for 3 min in 30-min intervals, as based on the net integer (NET INT) values of the LH response. Similar conclusions were published in papers where dynamic cell functions were more carefully analyzed (21).

(*iii*) Even the most potent antagonists to LHRH do not decrease the basal LH secretion provided precautions are made to prevent the effects of any specific or nonspecific stimulation of the cells at a time of the exposure. In view of our data, the real basal secretion of LH seems to be a "leakage"-like phenomenon, a result of nonspecific electrochemical events on the membrane, and independent of the specific LH-releasing mechanisms. This is why an indirect approach, such as measuring response to LHRH stimulation, is required for antagonist studies. Based on our data, we suggest that when LH antagonist activity was detected without specific stimulation either in static or in dynamic systems, the cells still might have been in a slightly stimulated condition. Testing LHRH antagonists on nonstimulated cells might lead to false-negative results.

(*iv*) The antagonistic LHRH analogues reduce and may even completely block the LHRH-induced LH response of the pituitary cells. The effect is temporary, dose dependent, and competitive. The antagonists affect only the specific LH release mechanism, apparently acting at the level of the receptors, since the analogues do not influence the LH-releasing potency of nonspecific stimuli such as potassium. The specificity of the effect is also supported by our (still unpublished) data, that our LHRH antagonistic analogues do not change the growth hormone-releasing hormone-induced growth hormone release and the basal or thyrotropin-releasing hormone-stimulated prolactin release.

(*v*) The size and the duration of the inhibitory effect on LH release depends on the sequence of administration of the antagonist and LHRH. Preincubation with the antagonist, before LHRH exposure, greatly increases the antagonistic activity. Ongoing LH release may also be reduced by administration of LHRH antagonist. This effect is similar to that produced by simultaneous administration of the antagonist with LHRH.

The differences in intensity and duration of the LH release-inhibiting activity may be explained by an apparent divergence between the association and dissociation rates of the various analogues to the LHRH receptors. The markedly different potency of the same analogue after preincubation or simultaneous administration with LHRH may also point out that the association rate of the analogues to the binding sites of the receptors is much higher than the dissociation rate. Once the analogue has been allowed to bind to the receptors without competition during the preincubation period, it is difficult to displace it by LHRH, which results in a greatly augmented inhibitory effect.

The slow onset of the inhibition of LH secretion in the case of SB-29 and the potentiating aftereffect of SB-102 can be explained by the presence of functionally inactive, nonspecific binding sites in the tissue. The release from the nonspecific sites may serve as a reserve for delayed receptor occupation and results in a delayed inhibitory effect of SB-29. On the other hand, SB-102 may show high affinity to some nonspecific binding sites and may reduce the nonspecific binding of LHRH during the subsequent exposure, resulting in higher specific stimuli. Previous studies indicated high- and low-affinity binding sites of rat pituitary membrane receptors for these antagonists (6, 22).

The evaluation of our results in conjunction with differences in binding parameters still remains to be completed. A full clarification will require the collection of more data on the interactions between the LHRH analogues and LHRH receptors. Following a receptor–ligand interaction, profound changes take place in membranes of living cells, which deeply affect the affinity and the number of the receptors on the surface of the cells within seconds (22–26). Such changes may not occur in isolated membrane preparations widely used for receptor studies, which could result in significant differences between the results of *in vivo* experiments and data collected by tests on isolated membranes (22–26). Since our aim is to elucidate the events that occur in living organisms, we consider the dynamic systems utilizing surviving cells to be promising for testing receptors. Superfusion methods might enhance our understanding of *in vivo* receptor interactions.

We are grateful to Dr. Sandor Bajusz for the synthesis of LHRH antagonists used in this study. We thank Dr. Kate Groot for her valuable help in performing the RIA determinations and the National Hormone and Pituitary Program of the National Institute of Diabetes and Digestive and Kidney Diseases (National Institutes of Health) for the gifts of the materials used in RIA. The work described in this paper was supported by National Institutes of Health Grants CA40003 and CA40004 (A.V.S.), by the Medical Research Service of the Department of Veterans Affairs (A.V.S.), and by the Hungarian Academy of Sciences Grant OTKA 2994 (V.C.).

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