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Real-Time Analysis of the Activities of GnRH and GnRH Analogs in αT3-1 Cells by the Cytosensor Microphysiometer

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Abstract Gonadotropin-releasing hormone (GnRH), acting via the GnRH receptor, elicited rapid extracellular acidification responses in mouse gonadotrope-derived αT3-1 cells as measured by the Cytosensor microphysiometer, which indirectly monitors cellular metabolic rates. GnRH increased the extracellular acidification rate of the cells in a dose-dependent manner ($EC_{50} = 1.81 \pm 0.24$ nM). The GnRH-stimulated acidification rate could be attenuated by protein kinase C (PKC) down-regulation, extracellular Ca^{2+} depletion, and the voltage-sensitive Ca^{2+} channel (VSCC) blocker nifedipine, indicating that the acidification response is activated by both Ca^{2+} and PKC-mediated pathways. Upon continuous exposure to 100 nM GnRH or periodic stimulation by 10 nM GnRH at 40 min intervals, homologous desensitization was more pronounced in the absence of extracellular Ca^{2+}, suggesting that desensitization of GnRH activity may be mediated via depletion of intracellular Ca^{2+} stores. We have also compared the potency of eight GnRH analogs on αT3-1 cells. No acidification response was detected for GnRH free acid, consistent with the idea that the C-terminal amide is a critical structural determinant for GnRH activity. Replacement of Gly-NH_2 at the C-terminus by *N*-ethylamide dramatically reduced the EC_{50} value, suggesting that substitution of the Gly-NH_2 moiety by *N*-ethylamide increases the potency of GnRH analogs. Substitution of Gly at position 6 by D-Trp significantly reduced the EC_{50} value, whereas D-Lys at the same position slightly increased the EC_{50} value, implying that either an aromatic amino acid or a non-basic amino acid at position 6 may be essential for potent GnRH agonists. In summary, our results demonstrate that the Cytosensor microphysiometer can be used to evaluate the actions of GnRH and GnRH analogs in αT3-1 cells in a real-time and noninvasive manner. This silicon-based microphysiometric system should provide new information on the structure-function studies of GnRH and is an invaluable tool for the screening of new GnRH agonists and antagonists in the future. J. Cell. Biochem. 80:304–312, 2001. © 2001 Wiley-Liss, Inc.

Key words: GnRH; GnRH analogs; GnRH receptor; desensitization; extracellular acidification rate

Gonadotropin-releasing hormone (GnRH) is a decapeptide which plays an essential role in the regulation of reproductive functions [Clayton and Catt, 1981]. Synthesized in the neurosecretory cells in the basal hypothalamus, GnRH is secreted to the portal circulation in a pulsatile manner in response to neural and neuroendocrine signals in the brain. Through interacting with the GnRH receptor in the gonadotropes of anterior pituitary, GnRH stimulates the synthesis and release of gonadotropins LH and FSH, which in turn stimulate gametogenesis and steroidogenesis in the gonads. Paradoxically, continuous exposure to pharmacological concentrations of GnRH causes a significant suppression in gonadal activity. This interesting phenomenon has opened up new avenues for the therapeutic use of GnRH analogs in the treatment of gonadal-steroid dependent tumors like prostate and breast cancers [Barbieri, 1992]. The antireproductive effects of GnRH analogs have also led to the development of GnRH agonists and antagonists as potential contraceptives [Corbin, 1982]. To date, several thousand of GnRH

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analogs have been synthesized and their biological activities characterized by various biological assays [Karten and Rivier, 1986]. The rapid exploration in the clinical use of GnRH analogs, however, necessitates an efficient screening system that can assess the potency of various GnRH analogs in vitro. In order to study the effect of GnRH analogs on pituitary gonadotropes, we have used the Cytosensor microphysiometer as the assay system and a mouse gonadotrope-derived αT3-1 cell line as the cell model. The Cytosensor microphysiometer employs a silicon-based sensor chip that can detect minute changes of pH in the extracellular environment [Parce et al., 1989; McConnell et al., 1992]. The principle of the Cytosensor microphysiometer is based on the fact that cellular metabolism produces acidic metabolites mainly in the form of CO_2 and lactic acid. Upon receptor activation and signal transduction initiation, the rate of excretion of these acidic metabolites or the extracellular acidification rate will be altered at a rate directly proportional to the cellular metabolic rate. Any changes in the acidification rate of the cells will then be detected by the sensor chip within the sensor chamber of the microphysiometer. In this report, we describe a real-time analysis of GnRH activity and a direct comparison on the potency of eight GnRH analogs in αT3-1 cells. Our results demonstrate that the Cytosensor microphysiometer is a rapid in vitro assay system to evaluate the activity of various GnRH analogs.

METHODS

Cell Culture and Reagents

αT3-1 cells were cultured in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) (Life technologies, Grand Island, NY). The cells were maintained at 37°C in 5% CO_2 incubator and passaged at weekly intervals. Phorbol 12-myristate 13-acetate (PMA) was from Calbiochem (La Jolla, CA). EGTA was from Sigma (St. Louis, MO) and nifedipine was from RBI (Natick, MA). Mammalian GnRH was purchased from Bachem (Bubendorf, Switzerland). GnRH analogs were purchased either from Bachem (GnRH free acid, GnRH (7–10), [D-Ala^6, *N*-methyl-Leu^7]-GnRH, [Des-Gly^{10}, D-Arg^6, Trp^7, Leu^8, Pro-NHEt^9]-GnRH, [Des-Gly^{10}, Pro-NHEt^9]-GnRH, [D-Trp^6]-GnRH) or Sigma ([Des-Gly^{10}, D-Ala^6, Pro-NHEt^9]-GnRH, [D-Lys^6]-GnRH).

Measurement of the Extracellular Acidification Rate

Extracellular acidification rates were measured by the Cytosensor microphysiometer as described previously [Ng et al., 1999]. Briefly, αT3-1 cells were seeded into sterile 12-mm capsule cups (Molecular Devices Corp., Sunnyvale, CA) at a density of 6×10^5 cells/capsule in DMEM supplemented with 10% FBS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) (Life technologies, Grand Island, NY). The cells were incubated at 37°C in 5% CO_2 incubator for 18 h. A spacer and a capsule insert (Molecular Devices Corp., Sunnyvale, CA) were placed in each capsule cup to define the internal size of the capsule at 50 μm high and 6 mm in diameter, and to trap the cells between two microporus polycarbonate membranes. The assembled capsule cups were placed in the sensor chambers of the microphysiometer. Bicarbonate-free DMEM or bicarbonate-free DMEM with 5 mM EGTA (Ca^{2+}-free DMEM) was used as a running medium. Due to omission of bicarbonate, each liter of the ruuning medium was supplemented by 11.1 ml of 4 M NaCl to preserve the osmotic balance. The running medium was pumped across the cells at a rate of 100 μl/min and the extracellular pH was continuously monitored during each 90 s interval. To measure the acidification rate, the pumps were momentarily stopped for 20 s to allow the accumulation of acidic metabolites within the sensor chambers. After the establishment of a basal acidification rate (about 1.5 h for αT3-1 cells), GnRH, GnRH analogs, or drugs diluted in the running medium was delivered to the sensor chambers via an alternative fluid pathway. The basal acidification rates from different sensor chambers were normalized to 100% and each of the peak acidification responses was expressed as a percentage of the basal rate before stimulation.

Statistical Analysis

All data were collected from eight independent sensor chambers ($n = 8$) and the values were expressed as mean ± SEM. Statistical analyses were performed using one-way ANOVA followed by Dunnett's test. Differences were considered to be significant at $P < 0.01$.

RESULTS AND DISCUSSION

Characterization of the GnRH-Stimulated Acidification Response

In this study, we took advantage of the real-time measurement of GnRH activity in αT3-1 cells by the Cytosensor microphysiometer. Early studies on ligand interaction with the GnRH receptor have been difficult since activation of the receptor is coupled to multiple second messengers, including inositol 1,4,5-trisphosphate, Ca^{2+}, and PKC [Horn et al., 1991; Huckle and Conn, 1988]. Therefore, multiple assays have to be performed in order to evaluate the post-receptor mechanisms following GnRH stimulation. The Cytosensor microphysiometer offers a new possibility for studying the interaction between GnRH and its receptor since it continuously monitors the extracellular acidification rate, which represents the summed effects of various signaling pathways that are activated or inhibited by the ligand. The flexibility of the system lies in that alterations in cellular metabolism and cellular acid excretion are general consequences of receptor activation. As a result, the Cytosensor microphysiometer can be employed as a single assay system to assess the functional coupling of receptors that are linked to multiple signal transduction pathways. In addition to the GnRH receptor, other members in the rhodopsin-like G protein-coupled receptor family including β-adrenergic [Owicki et al., 1990], dopaminergic [Chio et al., 1994], and muscarinic [Baxter et al., 1994] receptors were also demonstrated to be responsive in extracellular acidification rate measurement.

Since the Cytosensor measures a very small change in the extracelluar pH, it has a limitation in that it requires a running medium lacking buffers like Hepes and bicarbonate. The lack of bicarbonate in the running medium inevitably perturbs the acid-base balance of the cells, which may in turn alter cellular responses. Nevertheless, omission of bicarbonate in the running medium serves two important functions. First, by lowering the buffering capacity of the running medium, it increases the sensitivity of extracellular pH measurement. Second, by eliminating additional CO_2 supplied by bicarbonate, it reduces the chance of forming air bubbles within the sensor chambers, which will otherwise lead to unstable signals. To minimize the effect of transferring the cells to a medium without bicarbonate, prolonged equilibration time was given for the cells to adapt to the lightly buffered medium, until a steady basal acidification rate was reached (1.5 h for αT3-1 cells). The equilibration time may allow the cells to re-establish the acid-base balance before they were exposed to GnRH. Also, to preserve the osmotic balance, the omitted bicarbonate was substituted by NaCl in preparing the running medium used in this study.

In order to show that GnRH can elicit an acidification response in αT3-1 cells, the cells were challenged with increasing concentrations of mammalian GnRH from 1 pM to 100 nM for 6 min (Fig. 1). GnRH dose-dependently increased the extracellular acidification rate of the cells. At high concentrations of GnRH (10 and 100 nM), the resolution of the acidification responses was biphasic: a transient increase in the acidification rate was followed by a small plateau phase. The responses peaked at 1.5 min after the exposure and then decreased rapidly even in the continuous presence of the ligand, an observation which is likely due to homologous desensitization [Anderson et al., 1995; McArdle et al., 1995; Weiss et al., 1995]. The EC_{50} value of the GnRH-stimulated response was 1.81 ± 0.24 nM, which is comparable to a previous study based on the measurement of total inositol phosphate accumulation ($EC_{50} = 1.7 \pm 0.9$ nM) [McArdle et al., 1995].

To delineate the signaling components that contribute to the GnRH-stimulated acidification response, the PKC activator phorbol 12-myristate 13-acetate (PMA), Ca^{2+}-free medium, and the L-type voltage-sensitive Ca^{2+} channel (VSCC) blocker nifedipine were used. When the cells were pre-incubated with 100 nM PMA for 30 min to down-regulate the PKC signaling pathway, the GnRH-stimulated response was dramatically reduced to almost basal level (Fig. 2a). The response was also significantly decreased in Ca^{2+}-free medium or in the presence of 10 μM nifedpine (Fig. 2b,c). However, the cells could still generate a modest acidification response of 5% above basal, a result which may be attributed to intracellular Ca^{2+} stores and/or other signaling mechanisms independent of extracellular Ca^{2+}. Taken together, the data show that both PKC and extracellular Ca^{2+} are essential in mediating the acidification response of αT3-1 cells to

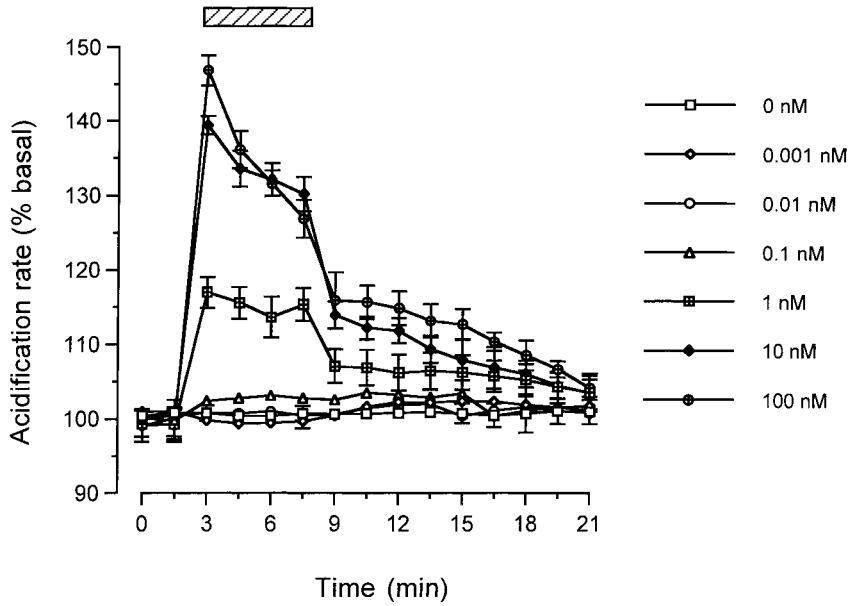

Fig. 1. Acidification responses of αT3-1 cells to increasing concentrations of GnRH. The cells were stimulated with increasing concentrations of GnRH for 6 min as indicated. The hatched bar represents the time during which the cells were exposed to GnRH. Values are the mean ± SEM of acidification rate measurement from eight independent sensor chambers (n = 8).

GnRH such that down-regulation of the PKC signaling pathway, removal of extracellular Ca^{2+}, or blockade of VSCCs dramatically reduces the GnRH-stimulated acidification response.

Desensitization of GnRH-stimulated Extracellular Acidification

Chronic or repetitive stimulation by high concentrations of a ligand is usually followed by a refractory period during which the cells are not responsive to further ligand stimulation, a phenomenon which is due to homologous desensitization. For G protein-coupled receptors, desensitization has been best characterized for the β-adrenergic family of receptors, in which receptor phosphorylation of intracellular amino acids at the C-terminus plays a major role in uncoupling the activated receptor from its G protein. Receptor phosphorylation is believed to enhance the binding of β-arrestin, which targets the desensitized receptor for internalization via clathrin-coated vesicles. Following internalization, receptors are either recycled back to the cell surface (resensitization) or degraded [Bouvier et al., 1988; Hausodorff et al., 1990; Lefkowitz et al., 1990]. Interestingly, the mammalian GnRH receptor, though lacking the entire C-terminal intracellular domain responsible for the phosphorylation of β-adrenergic [Hausodorff et al., 1990] and rhodopsin [Lorenz et al., 1991] receptors, elicits profound homologous desensitization in gonadotropes stimulated with high concentrations of GnRH or its agonists [Anderson et al., 1995; McArdle et al., 1995; Weiss et al., 1995]. It has been shown that desensitization of the mammalian receptor, unlike its non-mammalian counterparts and other receptors in the same family, occurred at a level distal to receptor and phospholipase C activation, which may involve inactivation of VSCCs and down-regulation of inositol 1,4,5-trisphosphate receptors [Heding et al., 1998; McArdle et al., 1996].

In this study, we analyzed, in real-time, the temporal profile of homologous desensitization of the GnRH receptor. We also studied the effect of extracellular Ca^{2+} depletion on the desensitization of the GnRH-stimulated acidification response. For the cells treated with Ca^{2+}-containing medium, continuous exposure to 100 nM GnRH caused a sharp increase (50%) in the acidification rate, but the spike reduced rapidly by about 15% after 20 min, indicating the occurrence of homologous desensitization (Fig. 3a). The response then maintained at a plateau of 35% above basal throughout the experimental period, suggesting that although homologous desensitization occurred at an early time frame after GnRH stimulation, it did not completely uncouple the receptor from its post-receptor signaling cascade. The maintenance of the acidification response after acute homologous desensitization is consistent with the observation that GnRH-stimulated LH secretion could be sustained during prolonged

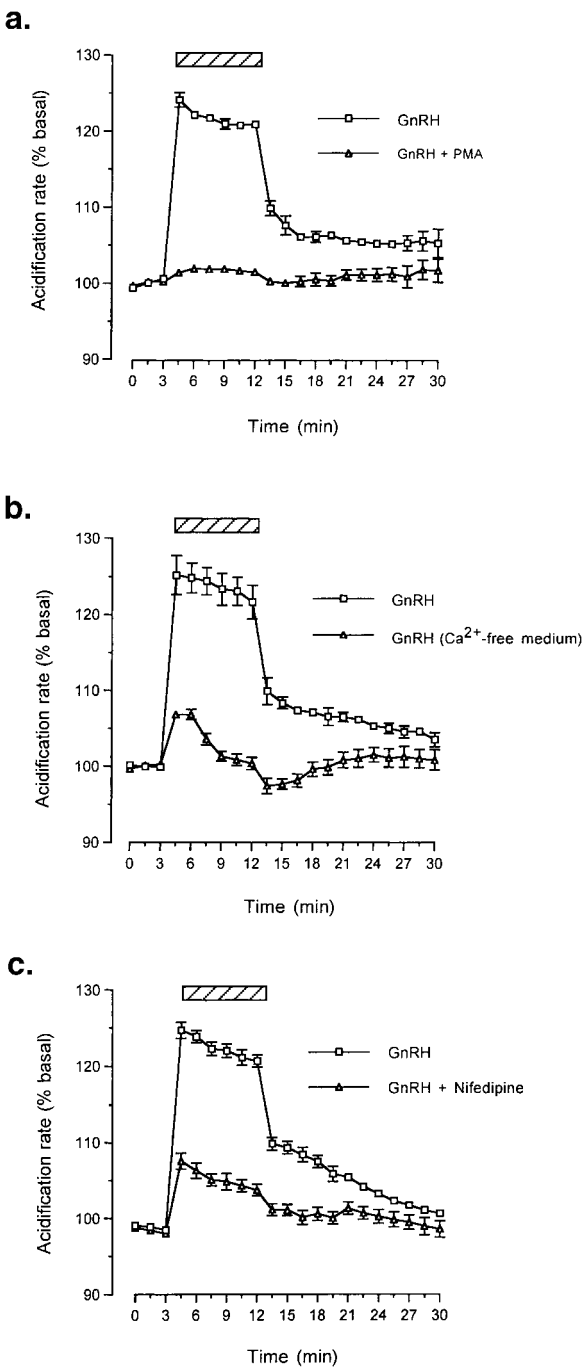

Fig. 2. Acidification responses of αT3-1 cells to 1 nM GnRH (**a**) in the presence (open triangle) or absence (open square) of 100 nM PKC activator, PMA, (**b**) in the presence (open square) or absence (open triangle) of extracellular Ca^{2+}, (**c**) in the presence (open triangle) or absence (open square) of 10 μM of the L-type VSCC blocker nifedipine. The hatched bars indicate the time during which the cells were exposed to GnRH. Values are expressed as the mean ± SEM of data from eight sensor chambers (n = 8).

GnRH exposure [Waters et al., 1992; Weiss et al., 1995]. For the cells treated with Ca^{2+}-free medium, homologous desensitization of GnRH activity was more prominent. Acute attenuation of the spike phase was also observed, but a smaller spike (30% above basal) was detected and the plateau phase declined linearly after 30 min of stimulation. Both the spike and plateau phases of the response were also reduced in the presence of 10 μM nifedipine. However, unlike that in the absence of extracelluar Ca^{2+}, the plateau phase did not attenuate significantly with time (Fig. 3a). This observation suggests the presence of additional components of extracellular Ca^{2+} influx that are insensitive to L-type Ca^{2+} channel blockers. These data indicate that the spike and plateau phases of the acidification response are dependent on extracellular Ca^{2+} influx, and the attenuation in both phases is most likely due to depletion of intracellular Ca^{2+} stores.

To further explore the role of extracellular Ca^{2+} depletion in the desensitization of the GnRH-stimulated acidification response, we tested the effect of repetitive GnRH stimulation on αT3-1 cells. In the presence of extracellular Ca^{2+}, repetitive exposures to 10 nM GnRH at 40 min intervals did not significantly alter the peak responses of the cells, implying that although homologous desensitization occurred within the time frame of the GnRH pulses, the cells could be resensitized, as sufficient recovery time was given (Fig. 3b). This observation conforms to the fact that a pulsatile GnRH release, but not a sustained release, is required to maintain normal pituitary functions. However, in the absence of extracellular Ca^{2+}, homologous desensitization was again more pronounced and the peak responses reduced from the first to the third GnRH pulse, a result which may also be attributed to the depletion of intracellular Ca^{2+} pools after repeated GnRH stimulation. This effect, however, was not clearly observed in the cells treated with Ca^{2+}-containing medium, since the slight decrease in the peak responses may be masked by the large acidification responses evoked in the presence of extracellular Ca^{2+}. Moreover, as the half-time for intracellular Ca^{2+} pool depletion and refilling was estimated to be less than 1 min [McArdle et al., 1996], the wash-out intervals between successive GnRH pulses (40 min) should provide enough time for the cells to replenish the intracellular Ca^{2+} stores from the

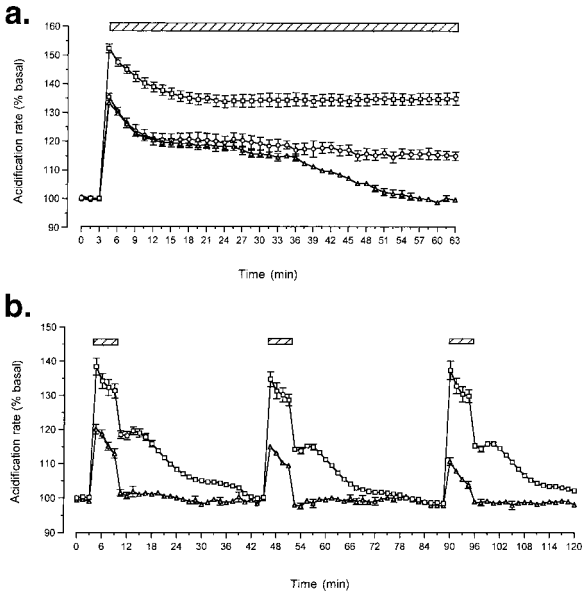

Fig. 3. Desensitization of the GnRH-stimulated acidification responses. (**a**) GnRH (100 nM) was given continuously for 60 min in the presence of Ca^{2+}-containing medium (open square), Ca^{2+}-free medium (open triangle), or 10 μM nifedipine (open circle) as indicated by the hatched bar. (**b**) GnRH (10 nM) in Ca^{2+}-containing medium (open square) or Ca^{2+}-free medium (open triangle) was given as three 6 min pulses as represented by the hatched bars. In between successive exposures to GnRH, the cells were washed with medium without GnRH for about 40 min. Values are the mean ± SEM of data from eight sensor chambers (n = 8).

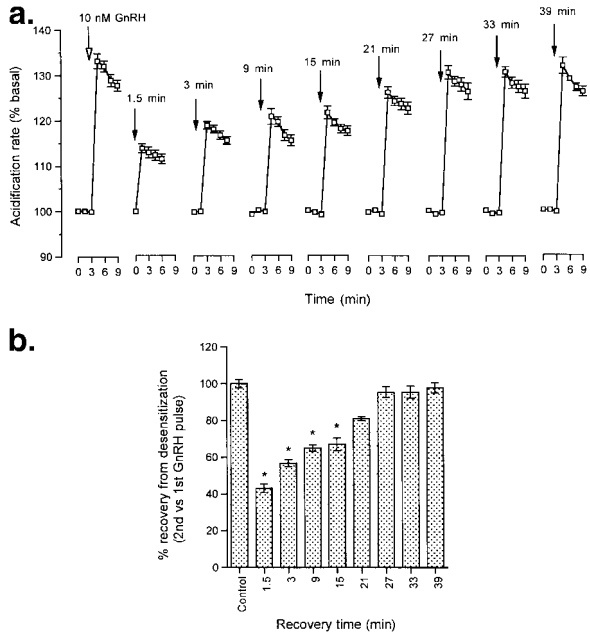

Fig. 4. Recovery of αT3-1 cells from desensitization. (**a**) αT3-1 cells were first stimulated by 10 nM GnRH for 6 min as illustrated by the white arrow. The second GnRH pulse was given for 6 min at different times after termination of the first GnRH pulse as indicated by the black arrows. The data shown are the mean ± SEM of data from eight sensor chambers (n = 8). (**b**) The percent recovery of the acidification response stimulated by the second GnRH pulse versus that of the first pulse (control). Bars bearing "*" are statistically different from the control ($P < 0.01$).

extracellular medium. In summary, our data indicate that desensitization of the GnRH-stimulated acidification response in αT3-1 cells is dependent on extracellular Ca^{2+}, and it is most likely mediated via depletion of GnRH-mobilizable Ca^{2+} pools.

Further attempts to define the time required for the cells to recover from desensitization were made by restimulating the cells with GnRH at various times after the first GnRH pulse (Fig. 4). The magnitude of the response elicited by the second GnRH pulse increased gradually with the duration of the recovery period. After transferring briefly to the running medium for 1.5 min, the cells could elicit 40% of the original response. After 21 min of recovery, the response to the second GnRH pulse was not different significantly from that of the first pulse ($P > 0.05$). Almost full recovery (> 95%) of the response was detected after 27 min, which lies within the range of GnRH pulse interval in vivo (about 30 min) [Urbanski et al., 1988].

Analysis of Structure-Activity Relationships of GnRH Analogs

The studies on the structure-activity relationships of various GnRH analogs have been a focus of interest owing to the fact that chronic exposure to high concentrations of GnRH agonists or antagonists can lead to suppression in reproductive functions and the growth of tumors. These phenomena have resulted in the clinical uses of GnRH analogs in the treatment of a variety of steroid-responsive tumors, and the development of GnRH agonists and antagonists as novel contraceptives [Barbieri, 1992; Corbin et al., 1982]. The ability of GnRH agonists and antagonists to suppress pituitary LH and FSH secretion and prevent premature ovulation has also been exploited in assisted reproduction programs like in vitro fertilization and gamete intrafallopian tube transfer [Barbieri and Hornstein, 1999].

In this study, the potency of GnRH and eight GnRH analogs in increasing the extracellular

TABLE I. Amino Acid Sequences of GnRH and GnRH Analogs

	1	2	3	4	5		6	7	8	9	10
GnRH, Mammal	pGlu	His	Trp	Ser	Tyr	-	Gly	Leu	Arg	Pro	Gly-NH_2
GnRH, Free acid	pGlu	His	Trp	Ser	Tyr	-	Gly	Leu	Arg	Pro	Gly-**OH**
GnRH (7–10)							H	Leu	Arg	Pro	Gly-NH_2
[Des-Gly^{10}, D-Arg^6, Trp^7, Leu^8, Pro-NHEt^9]-GnRH	pGlu	His	Trp	Ser	Tyr	**D**	**Arg**	**Trp**	**Leu**	Pro	**NHEt**
[Des-Gly^{10}, D-Ala^6, Pro-NHEt^9]-GnRH	pGlu	His	Trp	Ser	Tyr	**D**	**Ala**	Leu	Arg	Pro	**NHEt**
[Des-Gly^{10}, Pro-NHEt^9]-GnRH	pGlu	His	Trp	Ser	Tyr	-	Gly	Leu	Arg	Pro	**NHEt**
[D-Ala^6, *N*-methyl-Leu^7]-GnRH	pGlu	His	Trp	Ser	Tyr	**D**	**Ala**	**MeLeu**	Arg	Pro	Gly-NH_2
[D-Lys^6]-GnRH	pGlu	His	Trp	Ser	Tyr	**D**	**Lys**	Leu	Arg	Pro	Gly-NH_2
[D-Trp^6]-GnRH	pGlu	His	Trp	Ser	Tyr	**D**	**Trp**	Leu	Arg	Pro	Gly-NH_2

Differences between the mammalian GnRH and its analogs are typed in bold.

TABLE II. Maximal Acidification Responses of αT3-1 Cells and the EC_{50} Values of GnRH and GnRH Analogs

GnRH and Analogs	Maximal acidification Response (% basal ± SEM)	EC_{50} (nM ± SEM)
GnRH, Mammal	146.8 ± 2.1	1.81 ± 0.24
GnRH, Free acid	106.5 ± 1.1[a]	—
GnRH (7–10)	100.7 ± 0.4[a]	—
[Des-Gly^{10}, D-Arg^6, Trp^7, Leu^8, Pro-NHEt^9]-GnRH	149.3 ± 1.3	1.63 ± 0.12
[Des-Gly^{10}, D-Ala^6, Pro-NHEt^9]-GnRH	138.2 ± 0.8[a]	0.083 ± 0.003[a]
[Des-Gly^{10}, Pro-NHEt^9]-GnRH	139.7 ± 0.7[a]	0.075 ± 0.005[a]
[D-Ala^6, *N*-methyl-Leu^7]-GnRH	131.1 ± 1.9[a]	0.12 ± 0.03[a]
[D-Lys^6]-GnRH	136.5 ± 1.6[a]	2.18 ± 0.19
[D-Trp^6]-GnRH	147.0 ± 0.8	0.073 ± 0.007[a]

[a]Statistically significant change (ANOVA, $P < 0.01$, n = 8).

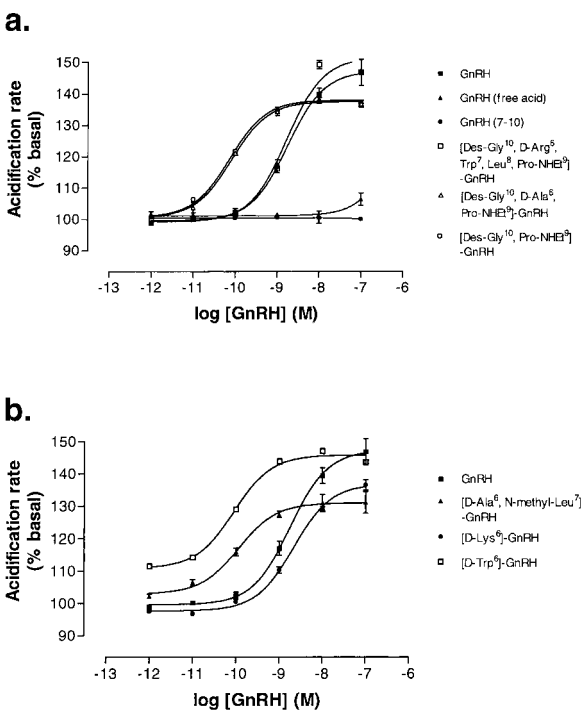

Fig. 5. Effects of GnRH analogs on the acidification responses of αT3-1 cells. (**a**) Dose-responses of αT3-1 cells to various GnRH agonists with modifications at the C-terminal domain. (**b**) Dose-responses of the cells to GnRH agonists with amino acid substitutions at position 6. Results are the mean ± SEM of data collected from eight independent sensor chambers (n = 8).

acidification rate of αT3-1 cells was compared. The structures of the GnRH analogs used were shown in Table I, and their respective maximal responses and EC_{50}values were summarized in Table II. The amino acid substitutions and structural modifications of the GnRH agonists are mostly at position 6 and the C-terminus, whereas the GnRH antagonists usually have substitutions at the N-terminus [Janecka et al., 1994]. The roles of individual amino acids in GnRH for receptor binding and activation have been extensively evaluated. It is now well accepted that both the N-terminal domain (pGlu-His-Trp-Ser) and the C-terminal domain (Pro-Gly-NH_2) are responsible for receptor binding, whereas the N-terminal domain is involved in receptor activation [Karten and Rivier, 1986; Millar et al., 1987; Sealfon et al., 1995].

Among the five agonists which have substitutions at the C-terminal domain (Fig. 5a), GnRH free acid elicited no acidification response, indicating that the terminal amide is a crucial structural determinant for GnRH activity. GnRH (7–10), which only contains the last four amino acids of the native peptide, was also without effect, showing that the N-terminal domain should be present in bioactive GnRH

analogs. Replacement of the Gly-NH_2 group by *N*-ethylamide (NHEt) significantly reduced the EC_{50} values of the GnRH analogs ([Des-Gly^{10}, D-Ala^6, Pro-NHEt^9]-GnRH, 22.1-fold, $P<0.01$; [Des-Gly^{10}, Pro-NHEt^9]-GnRH, 24.1-fold, $P<0.01$), suggesting that the Gly-NH_2 group is not critical for GnRH activity and the substitution of which by *N*-ethylamide increases the potency of GnRH analogs, possibly by enhancing their binding affinity. Earlier studies also found that substitution by ethylamide or propylamide in this position could enhance the potency of GnRH analogs [Coy et al., 1975; Fujino et al., 1992]. However, it is interesting to note that the maximal acidification responses induced by the two analogs with NHEt substitution ([Des-Gly^{10}, D-Ala^6, Pro-NHEt^9]-GnRH and [Des-Gly^{10}, Pro-NHEt^9]-GnRH) were lower than that of GnRH (Table II). These observations suggest that although the terminal Gly-NH_2 group is not essential for high potency, it may play a role in determining the efficacy of GnRH analogs. The salmon GnRH analog ([Des-Gly^{10}, D-Arg^6, Trp^7, Leu^8, Pro-NHEt^9]-GnRH), which has substitutions at positions 6, 7, and 8, was found to be equipotent to mammalian GnRH (Fig. 5a).

The potency of GnRH analogs with substitutions at position 6 was also compared (Fig. 5b). It has been postulated that the presence of Gly in this position allows for the flexibility and maintenance of the proposed conformation of type-II β turn for GnRH [Monahan et al., 1973]. Thus more bulky amino acid substitutions like Ile, Val, and Ala analogs were found to have low activity [Coy et al., 1973; Monahan et al., 1973]. Since it was later discovered that D-amino acid substitutions could favor the formation of the active conformation and hence the binding affinity of GnRH, numerous GnRH superagonists with D-amino acid substitutions at position 6 have been synthesized [Karten and Rivier, 1986]. To investigate the effect of different side chains on the agonist activity of analogs with D-amino acid substitutions, 3 GnRH analogs with various amino acids (aromatic, basic, and neutral) at position 6 were studied. Substitution of Gly at position 6 by aromatic D-Trp ([D-Trp^6]-GnRH) dramatically reduced the EC_{50} value (24.8-fold, $P<0.01$), whereas substitution at the same position by basic D-Lys did not significantly change the EC_{50} value. Substitution by a neutral amino acid D-Ala ([D-Ala^6, *N*-methyl-Leu^7]-GnRH) also reduced the EC_{50} value by 11.3-fold ($P<0.01$) (Table II). Therefore, our data suggest that either an aromatic or a non-basic D-amino acid at this position may be required for potent GnRH agonists. For [D-Ala^6, N-methyl-Leu^7]-GnRH, it has been proposed that the N-methyl-Leu at position 7 serves to eliminate the hydrogen bond between the C=O group of Ser and NH group of Leu in the establishment of a type-II β turn conformation of GnRH [Ling and Vale, 1975]. Previous data showed that D-Ala^6 substitution dramatically increased GnRH activity by about 400% [Monahan et al., 1973], but substitution of Leu^7 with *N*-Me-Leu had no change in activity [Ling and Vale, 1975]. Thus, it is likely that the enhanced potency observed in [D-Ala^6, *N*-methyl-Leu^7]-GnRH is due to the influence of D-amino acid substitution at position 6, and this effect is independent on the formation of a stabilized hydrogen bond between Ser^4 and Leu^7.

In conclusion, we have demonstrated, based on acidification rate measurement, the GnRH-stimulated acidification response is dependent on PKC and extracellular Ca^{2+}. Continuous or repetitive stimulation by high concentrations of GnRH elicits homologous desensitization, which is most likely due to depletion of intracellular Ca^{2+} pools. Analysis of GnRH analogs revealed that replacement of Gly-NH_2 group by *N*-ethylamide or substitution of Gly by D-Trp at position 6 could increase the potency of GnRH agonists.

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