V. S. Ananthanarayanan
O. Salehian
K. S. Brimble

Interaction of gonadotropin-releasing hormone and its agonist analogs with Ca^{2+} in a nonpolar milieu. Correlation with biopotencies

Authors' affiliations:
V. S. Ananthanarayanan, O. Salehian, and K. S. Brimble, Department of Biochemistry, McMaster University, Hamilton, Ontario, Canada L8N 3Z5.

Correspondence to:
Professor *V.S. Ananthanarayanan*
Department of Biochemistry
McMaster University
1200 Main Street West
Hamilton, Ontario
Canada L8N 3Z5
Phone: (905) 529-7070, ext. 22783
Fax: (905) 522-9033
E-mail: ananth@mcmaster.ca

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Abstract: Extracellular Ca^{2+} is necessary for the action of gonadotropin-releasing hormone (GnRH). Assuming that this partly because of the interaction of the hormone with the relatively abundant extracellular Ca^{2+} in the low dielectric milieu of the bilayer plasma membrane, we studied the interaction of GnRH and five of its agonist analogs with Ca^{2+} under membrane-mimetic conditions. The peptides used, in increasing order of their reported gonadotropin-releasing activities, were: des-amide GnRH (or GnRH-OH); [Ala^6]GnRH; [D-Ala^6]GnRH; des-Gly^{10}[D-Ala^6,Pro^9-NHEt]GnRH and, des-Gly^{10}[D-Trp^6,Pro^9-NHEt]GnRH. Changes in the far-UV CD and fluorescence spectra of these peptides in trifluoroethanol were used to monitor conformational changes and obtain the Ca^{2+}-binding isotherms. The data show that GnRH and its active analogs contain two Ca^{2+} binding sites, whereas the inactive analogs have only one. The extent of Ca^{2+} binding by the agonist peptides paralleled their biological potency ranking. The superactive analog des-Gly^{10}[D-Trp^6,Pro^9-NHEt]GnRH exhibited the ability to transport Ca^{2+} ions across large unilamellar vesicles of dimyristoylphosphatidylcholine. Our study shows that significant differences among the GnRH and its analog peptides, suggestive of differences in their conformations, are manifested only in the presence of Ca^{2+}. This observation would provide a basis for understanding GnRH action in terms of the hormone's interaction with Ca^{2+} in the lipid milieu.

Gonadotropin-releasing hormone (GnRH; sequence, pGlu^1-His^2-Trp^3-Ser^4-Tyr^5-Gly^6- Leu^7-Arg^8-Pro^9-Gly^{10}-NH_2) stimulates the release of gonadotropin and follicle-stimulating hormone from the pituitary gland and has found extensive therapeutic use as a regulator of ovulation (1). The design of potent GnRH analogs therefore has been the target of intense

research during the past three decades. Several thousands of agonist and antagonist analogs of the hormone have been synthesized and used in pharmacological studies (2–4), and much progress has been made in delineating the biochemical aspects of GnRH action. The hormone binds with high affinity to a 50- to 60-kDa plasma membrane receptor which recently was cloned and shown to be a member of the seven-transmembrane-helix G-protein-coupled receptor family (5). The activation of GnRH receptor leads to an increase in cytoplasmic Ca^{2+} and the synthesis and release of gonadotropin (6). Ca^{2+} ionophores that increase intracellular Ca^{2+} cause gonadotropin release with an efficacy similar to that of GnRH (7). That extracellular Ca^{2+} is necessary for mediating GnRH action has been known for a long time (8, 9), although the nature and extent of its involvement are not fully understood (10). Na^{+} or Mg^{2+} cannot substitute and abolish the Ca^{2+} requirement (8).

Numerous studies have been devoted to evaluating the consequences of changes in the amino acid sequence of GnRH on its biological potency. Some of the important results of these structure-activity relation studies (reviewed in Refs. 2–4 and 11) are: a) No single residue is essential for activity; b) the N-terminal domain, p-Glu^1-His^2-Trp^3, and the C- terminal domain, Pro^9-Gly^{10}-NH_2, are important in receptor binding activation; and c) the central domain, Tyr^5-Gly^6-Leu^7-Arg^8, is important for biological potency. Replacement of Gly^6 by L-amino acids like Ala, Ile or Pro leads to weak agonists with drastically reduced (25–1000 times less) potencies, whereas replacement by D-amino acids in this position, such as D-Ala, D-Trp or D-Pro, results in "superagonists" having 2- to 100-fold increased potency relative to the native hormone.

As a prerequisite to understanding of GnRH action and design of potent agonists and antagonists for therapeutic use, much effort has been directed toward the elucidation of the conformation of the hormone and its analogs and relating this information to the observed biological activities of these analogs. Spectroscopic data including circular dichroism (CD) (12) and nuclear magnetic resonance (NMR) (13–15) have indicated a highly flexible conformation for GnRH in water and other polar solvents whereas, in nonpolar solvents such as trifluoroethanol (TFE), the presence of ordered structures has been detected by CD (16, 17). Theoretical computations of GnRH structure (11, 18–20) have identified a type II′ β-turn in the middle region (Tyr^5-Gly^6-Leu^7-Arg^8) of the hormone and have implicated this conformation as a requirement for receptor binding. The latter suggestion finds support in the enhanced potencies of GnRH analogs containing D-amino acids in position 6 which may stabilize the β-turn conformation as suggested by Monahan *et al.* in 1973 (21). Several cyclic peptide derivatives of GnRH intended to further stabilize this β-turn have been designed (22) but were found to have only antagonist but not agonist activities. Extensive computational (23) and NMR (24, 25) analyses of these cyclic analogs have verified the expected stabilization of the Gly^6-Leu^7 β-turn. However, conformational flexibility was noted even in the cyclic molecules, particularly between residues 1 and 4 (11).

The inherent flexibility of GnRH and its analogs revealed by these studies makes it difficult to distinguish the most and least active peptides by physicochemical techniques. However, as pointed out recently by Sealfon *et al.* (11), such data are essential to understand the nature of the bioactive conformation (i.e. the structure recognized by the receptor) of GnRH and to obtain useful structure-activity ranking among agonists and antagonists. Structural comparison of hormone analogs with agonist activity are, in fact, more useful than study of antagonists in understanding a hormone's bioactive conformation, because the agonists not only bind to the receptor but lead to its activation and to downstream events. In this study, we have sought to obtain an insight into the conformational differences among GnRH and a selected set of agonist analogs by examining their interaction with Ca^{2+} in TFE. The basis for our approach lies in the possibility that GnRH, like other peptide hormones (26), may assume structures quite different from those in an aqueous medium when they are in the low-dielectric milieu of the bilayer lipid membrane where it would interact with its receptor. In addition, the hormone, in this milieu, may interact with Ca^{2+} (27) which is relatively abundant in the extracellular medium and is important for GnRH action (8–10). The results reported here show significant differences among GnRH and its analogs in their Ca^{2+}-binding behaviors which correlate very well with the relative activities of these peptides.

Experimental Procedures

Materials

Arsenazo III, ethylenediaminetetraacetic acid (EDTA), GnRH, melittin, TFE and N-[hydroxy-ethyl]piperazine-N′-[2-ethanesulfonic acid] (Hepes) were obtained from Sigma Chemical Co. (St. Louis, MO). Triton X-100 was from Pierce Chemical Co. (Rockford, IL). A23187, 8-aminonaphthalene-1,3,6-trisulfonic acid, disodium salt (ANTS) and *p*-xylene-bis-pyridinium bromide (DPX) were from Molecular Probes (Eugene, OR). desGly^{10}[D-Trp^6,Pro^9-NHEt]GnRH and GnRH-OH (free acid) were generous gifts from M.J. Karten (National Institutes of Health, Baltimore) and J. Rivier (Salk

Institute, San Diego). [D-Ala^6]GnRH, [Ala^6]GnRH and des-Gly^{10}[D-Ala^6,Pro^9-NHEt]GnRH were purchased from Bachem Inc. (Torrance, CA). $Ca(ClO_4)_2$ and $Mg(ClO_4)_2$ were purchased from GFS Chemicals (Columbus, OH), and DMPC was from Avanti Polar Lipids (Alabaster, AL).

Methods

Aqueous solutions were made with water purified using a Nanopure water purification system (Barnstead, IA) to ensure minimal levels of divalent cations. TFE was distilled to minimize fluorescing impurities that develop on storage. Concentrations of GnRH, analogs and fragments containing tryptophan were determined from absorbance in 98% TFE using an extinction coefficient of 629.5 $M^{-1}cm^{-1}$ at 295 nm as determined in our laboratory. All peptides were >95% pure as reported by the supplier and as judged from reversed-phase high-performance liquid chromatography using a C-18 column. They were dried under high vacuum before use and were stored under argon in desiccated containers to ensure minimal water content. The perchlorate salts were lyophilized and kept under high vacuum to minimize water content which was assessed from their NMR spectra in TFE. Lipids were kept under argon to prevent oxidation which, in turn, caused difficulty in preparing leak-free unilamellar vesicles.

Circular dichroism

CD spectra were collected at ambient temperature (~22°C) using a computer-controlled Jasco J- 600 spectropolarimeter. The peptide concentration varied from 30 to 50 μM. One-millimeter and 1-cm quartz cells were used, respectively, in the far- and near-UV regions. Spectra were signal-averaged over 8–16 scans. For metal ion titrations, 10-μL aliquots of a stock solution of the perchlorate salt in TFE were added to the peptide solution in the cell with mixing to yield the required final metal ion concentration. The mean-residue ellipticity [θ] was expressed in deg cm^2 $dmol^{-1}$.

Fluorescence spectroscopy

Fluorescence spectra were recorded on a Perkin-Elmer LS 50 spectrofluorometer at ambient temperature (~22°C) using 0.5- and 1-cm rectangular quartz cuvettes. Peptide concentrations were close to those used in the CD experiments. Metal ion titration was carried out as in the CD experiments and mixing was done using a built-in magnetic stirrer. Tryptophan fluorescence was monitored by setting the excitation wavelength at 280 nm and the emission wavelength at 340 nm. The slit widths were 5 nm. Solvent baseline was subtracted routinely from the sample spectra.

Ca^{2+} translocation

Large unilamellar vesicles (LUV) of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) containing trapped Arsenazo III were made as described previously (28). The change in absorbance at 650–700 nm of the trapped Arsenazo III caused by Ca^{2+} influx into the LUVs was monitored at 37 ± 1°C (maintained by circulating water from a Lauda constant temperature bath) at different time intervals using a Perkin-Elmer Lambda-6 UV/VIS spectrophotometer with a 1-nm slit width and a 300 nm min^{-1} scan speed. The sample and reference cuvettes initially contained 750 μL of the buffer containing 50 mM Hepes, pH 7.4, and 125 mM KCl , 100 μL of the LUV suspension (containing 3.5 μmol lipid) and 30 μLof 100 mM $CaCl_2$ (final Ca^{2+} concentration of 3.4 mM). Difference spectra produced by peptide-mediated Ca^{2+} influx in the sample cuvette were recorded between 400 and 700 nm at specified times. EDTA was added at the end of the experiment to give a final concentration of 5 mM to estimate the amount of the dye which had leaked out. The difference absorbance change (ΔOD) at any given time was converted to the amount of Ca^{2+} translocated as described previously (28, 29).

Assessment of membrane integrity

Peptide-induced perturbation, if any, of the bilayer membrane (other than that caused by genuine ion transport) was assessed using the procedures described earlier (29). The first involved the measurement of the peptide-induced leakage of the fluorescent dye ANTS trapped inside LUVs along with the quencher DPX (30), any such leakage resulting in an increase in the fluorescence of ANTS. The test for peptide-induced membrane fusion also involved trapping the two dyes above independently in two sets of LUVs so that membrane fusion would cause a reduction in ANTS fluorescence (30).

Results

Spectral changes on Ca^{2+} binding

CD and fluorescence spectroscopy were used to monitor the binding of Ca^{2+} and also to obtain some information on the structural alteration in the backbone and aromatic side-chains caused by the binding. The CD spectrum of GnRH in TFE in the far-UV region (between 210 and 240 nm) displayed a negative band with a minimum at 218 nm (Fig. 1). Comparison with reported spectra for known secondary structures (31) suggested a predominantly β-turn or β-structure for GnRH in this solvent. The β-turn is consistent with suggestions from earlier spectral studies (11–17) and theoretical calculations (11, 18–

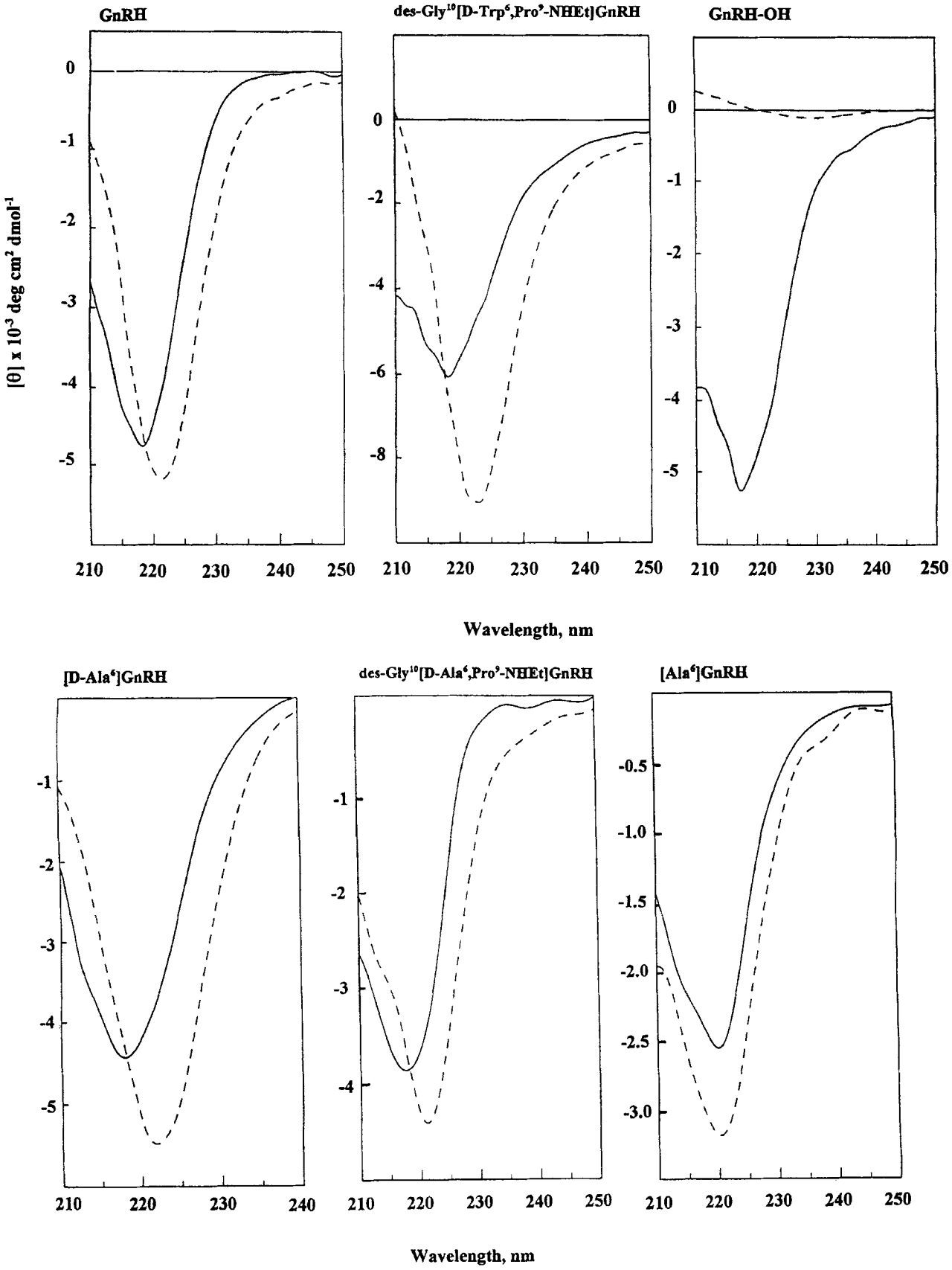

Figure 1. CD spectra of GnRH and agonist peptides in TFE. Peptide concentrations were between 45 and 55 μM. In each box, the solid curve corresponds to the free peptide and the dashed curve to peptide treated with 2 molar excess of $Ca(ClO_4)_2$.

20). The CD spectrum was concentration-independent in the range used (25–50 μM) in this study, hence any intermolecularly associated structures are unlikely to be significantly populated. However, the spectrum did exhibit significant concentration dependence above about 75 μM, and it is possible that GnRH adopts the β-structure above this concentration in TFE as found in the earlier CD studies in this solvent (16, 17). The CD spectra of the five agonist analogs of GnRH were qualitatively similar to that of the parent hormone (Fig. 1). The ellipticity [θ] of the negative CD band at 218 nm lay between 3500 and 5000 deg cm^2 $dmol^{-1}$ for all but the L-Ala^6 analog, which had a $[\theta]_{218}$ of about 2200. From the CD data one might conclude that the structures adopted by GnRH and its analogs are essentially similar.

The addition of Ca^{2+} resulted in a progressive increase in the size of the negative CD band in all cases except GnRH-OH until saturation was achieved. The magnitude of this increase varied with the peptide as did the concentration of Ca^{2+} required to achieve the maximal CD change. The CD spectra of the peptides at a Ca^{2+}/peptide molar ratio of two are shown in Figure 1. With GnRH-OH, in contrast, the ellipticity around 230 nm became more negative up to about one molar equivalent of Ca^{2+} beyond which it became progressively less negative (Fig. 1) and, at Ca^{2+}:peptide ratios greater than two, the ellipticity became positive in sign. Figure 2 shows a plot of the fractional change in the ellipticity of the negative CD band against the Ca^{2+}/peptide ratio for each of the peptides except GnRH-OH. The fractional ellipticity change is defined as $\{[\theta]_{obs} - [\theta]_{min}\}/\{[\theta]_{max} - [\theta]_{min}\}$, where $[\theta]_{obs}$ corresponds to the observed ellipticity at a given Ca^{2+} concentration, and $[\theta]_{min}$ and $[\theta]_{max}$ represent, respectively, ellipticities at the start and end of the Ca^{2+} titration. The Ca^{2+} titration for GnRH-OH did not show a leveling off the ellipticity at high Ca^{2+}/peptide ratios so that $[\theta]_{max}$ could not be determined. The titration data for this peptide are shown in the inset of Figure 2 as a plot of [θ] at 230 nm against the Ca^{2+}/peptide ratio.

The Ca^{2+} binding curves for GnRH and the three potent agonists [D-Ala^6]GnRH, des- Gly^{10}[D-Trp^6,Pro^9-NHEt]GnRH and des-Gly^{10}[D-Ala^6,Pro^9-NHEt]GnRH show that these peptides bind two Ca^{2+} ions (Fig. 2). The binding of the first ion induces less spectral (and hence conformational) change than that of the second ion. On the other hand, the weakly active L-Ala^6 peptide bound only one Ca^{2+} ion which caused a rather large relative change in the CD spectrum. In contrast to these peptides, the inactive free acid GnRH-OH exhibited a markedly different behavior (Fig. 2, inset (a)). This peptide showed qualitatively similar CD changes for the binding of the first Ca^{2+} as the other peptides, but further addition of

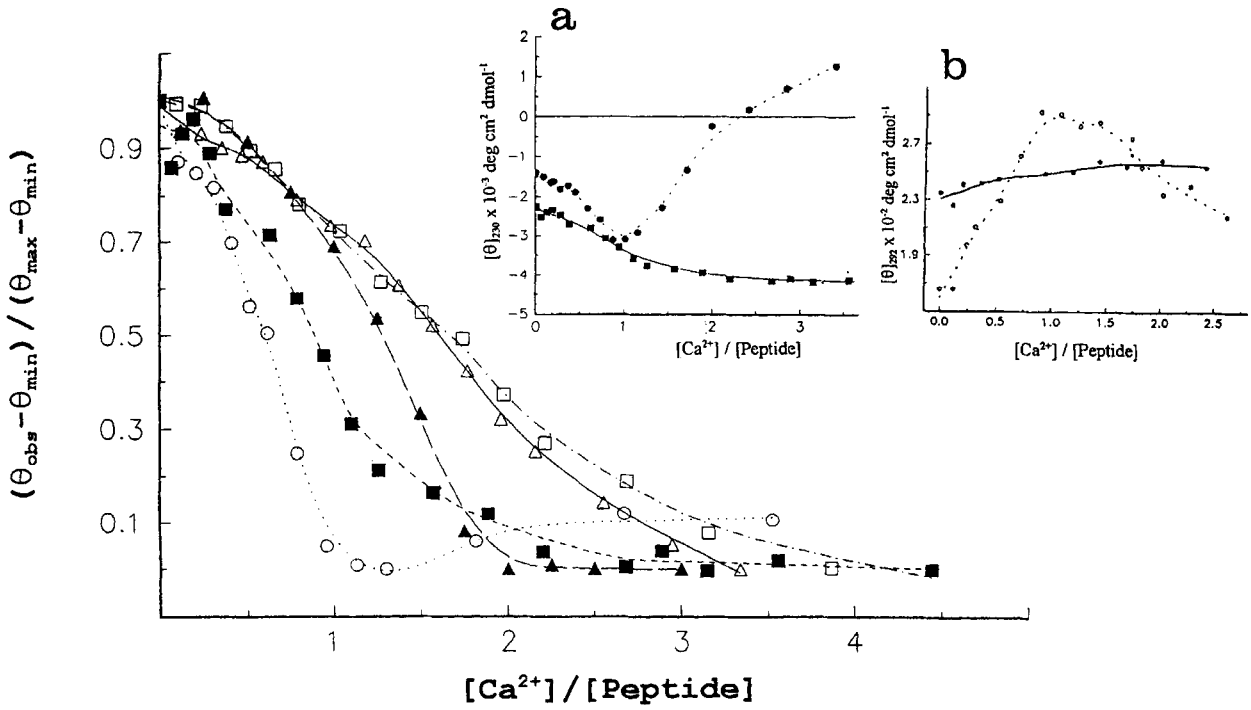

Figure 2. Ca^{2+} binding isotherms of GnRH and agonist peptides obtained from CD data in TFE at ambient temperature (~22°C): GnRH (■), [Ala^6]GnRH (○), [D-Ala^6]GnRH (▲), des-Gly^{10}[D- Ala^6, Pro^9-NHEt]GnRH (△) and [des-Gly^{10},D-Trp^6, Pro^9-NHEt]GnRH (□). Peptide concentrations were between 45 and 55 μM. θ_{max}, θ_{min} and θ_{obs} represent, respectively, ellipticity values at 220 nm ($[\theta]_{220}$) obtained at the lowest, highest and intermediate $[Ca^{2+}]$/[peptide] ratios. Insets: (**a**) Plot of $[\theta]_{230}$ of GnRH-OH (●) and, for comparison GnRH (■) against the $[Ca^{2+}]$/[peptide] ratio; and (**b**) Changes in tryptophan ellipticity, $[\theta]_{292}$, on Ca^{2+} addition to GnRH (●) and [des-Gly^{10},D-Trp^6,Pro^9-NHEt]GnRH (○).

Ca^{2+} caused a reversal of this change suggestive of the onset of a different type of Ca^{2+}-peptide complex. However, there was no saturation of the binding. The data thus suggest a nonspecific electrostatic interaction beyond the first Ca^{2+} binding in GnRH-OH (see "Fluorescence Data").

Structural changes in the peptides caused by Ca^{2+} binding in TFE also were gleaned from their CD spectra in the near-UV region between 250 and 310 nm. The weak bands around 280 and 290 nm in GnRH arising from asymmetrically placed Tyr and Trp residues, respectively, as observed earlier by Cann *et al.* (12) became more positive on Ca^{2+} addition (data not shown). These changes were relatively small in magnitude. However, they were clearly visible and were more prominent in the superagonist des-Gly^{10}[D-Trp^{6}, Pro^{9}-NHEt]GnRH than in GnRH as may be seen from the titration curves constructed from the tryptophanyl CD changes which are shown in inset (b) in Fig. 2. In the former peptide, the spectral changes on binding the first and the second Ca^{2+} ions are distinct and suggest changes in the asymmetric placement of the Trp residue in the respective complexes compared with the native hormone.

Addition of Mg^{2+} and Zn^{2+} (in the form perchlorate salts) to GnRH and des-Gly^{10}[D- Trp^{6},Pro^{9}-NHEt]GnRH caused the negative far-UV CD spectral band around 218 nm to decrease rather than increase in its magnitude seen earlier with Ca^{2+}, the effect of Zn^{2+} being much smaller than that of Mg^{2+} (data not shown). However, the subsequent addition of Ca^{2+} to a Mg^{2+}- or Zn^{2+}-treated peptide reverted the spectrum to that produced by Ca^{2+} alone (data not shown), implying that Ca^{2+} competes these other ions out of the metal ion binding sites in these peptides. No CD spectral changes were observable on adding severalfold excess of $NaClO_4$ to GnRH in TFE. This indicates that metal ion binding to the hormone is not a nonspecific electrostatic phenomenon.

Fluorescence data

Ca^{2+}-induced changes in the conformation of GnRH and the analog peptides in TFE also were monitored by the fluorescence emission of the Trp residue at 340 nm. Although there was no significant shift in the wavelength of the emission maximum, the fluorescence decrease progressively on Ca^{2+} addition (Fig. 3, inset), possibly because of the ap-

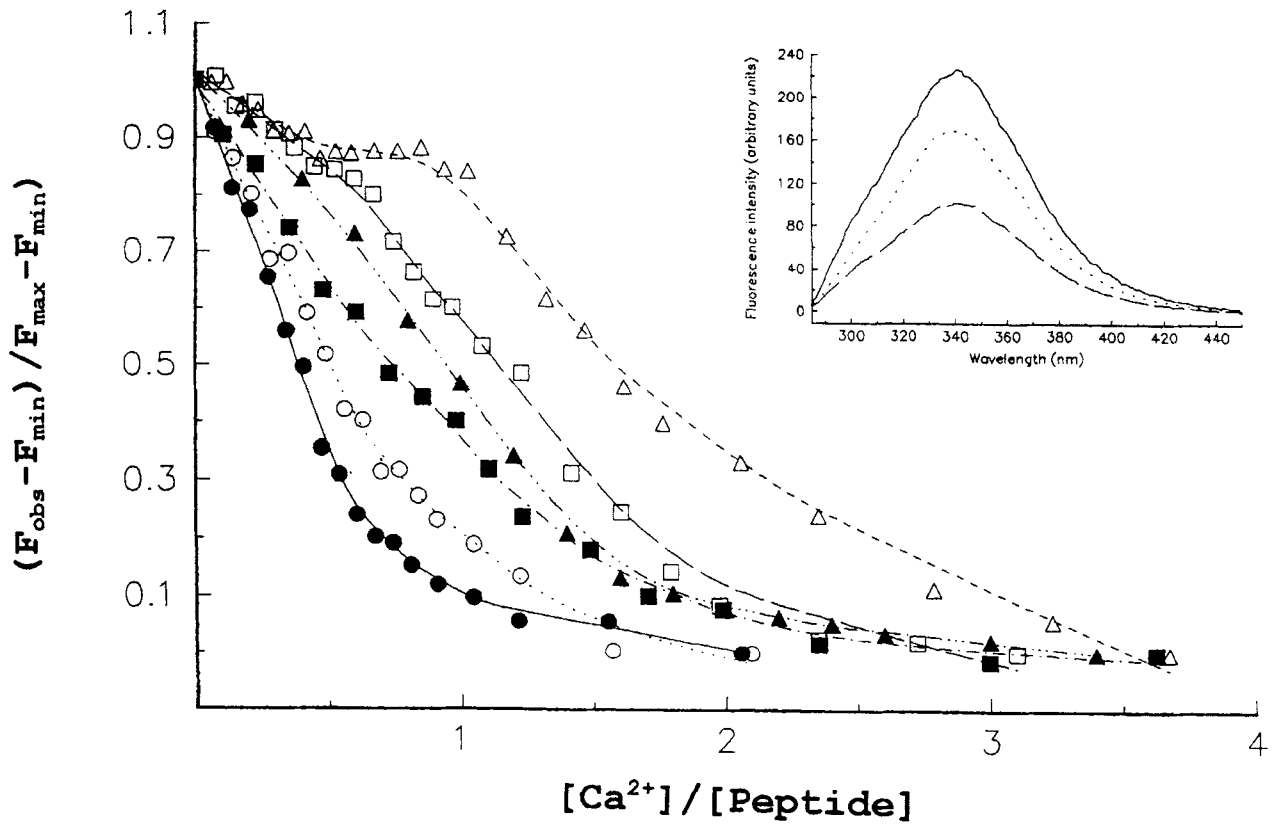

Figure 3. Ca^{2+}-binding isotherms of GnRH and agonist peptides in TFE at ambient temperature (~22°C) obtained from fluorescence data: GnRH (■), GnRH-OH (●), [D-Ala^{6}]GnRH (▲), [Ala^{6}]GnRH (○), [des-Gly^{10},D-Trp^{6}, Pro^{9}-NHEt]GnRH (□) and des-Gly^{10}[D-Ala^{6},Pro^{9}-NHEt]GnRH (△). Peptide concentrations were 30 to 40 μM. F_{max}, F_{min} and F_{obs} represent, respectively, tryptophan emission intensities at 340 nm (after excitation at 275 nm) measured at the lowest, highest and intermediate $[Ca^{2+}]$/[peptide] ratios. Inset: Tryptophan fluorescence emission spectra in TFE of: GnRH (—), GnRH with one molar excess of $Ca(ClO_4)_2$ (...) and GnRH with two molar excess of $Ca(ClO_4)_2$ (---).

proach of quenchers like the Asp or His residues as observed with glucagon (32). Unlike its peculiar CD spectral behavior, Ca^{2+} binding by GnRH-OH produced fluorescence changes qualitatively similar to the other peptides so that all of the binding isotherms could be displayed in a similar way by plotting $\{(F_{obs} - F_{min})/(F_{max} - F_{min})\}$ against the molar ratio of Ca^{2+} to peptide. The binding isotherms shown in Fig. 3 are remarkably similar in their features to those obtained from CD measurements (Fig. 2) in terms of the stoichiometry and, with one exception, in terms of the relative displacement of the curves along the Ca^{2+}/peptide ratio axis. The exception was the placement of the binding curve of des-Gly^{10}[D-Ala^6,Pro^9-NHEt]GnRH on the right side of des-Gly^{10}[D-Trp^6,Pro^9-NHEt]GnRH (Fig. 3) unlike the CD data where these were very close to each other (Fig. 2). This discrepancy may be caused by a possible fluorescence energy transfer between the Trp residues and Tyr^5 in the latter peptide; any such effect was not taken into account while normalizing the fluorescence increase shown in Fig. 3. Compared with the CD data, the biphasic nature of the binding (indicative of two independent binding sites) is more obvious in the fluorescence data particularly in the case of the superagonist analogs. In view of the uncertainty about the interaction between the binding sites, the overall binding affinity of the peptides toward Ca^{2+} could only be estimated roughly. These were about 10^5 M^{-1} based on the peptide concentration (25–50 μM) needed to observe the CD and fluorescence spectra of the Ca^{2+} complexes.

Ca^{2+} translocation studies

In our earlier studies (29, 32, 33) we examined the interaction of peptide hormones with Ca^{2+} both in terms of their binding the cation in the low-dielectric TFE as well as their ability to bind the cation within the lipid bilayer of synthetic liposomes. In the latter case, depending on their Ca^{2+} binding affinity and hydrophobicity, the hormones would translocate the ion across the lipid bilayer via a carrier mechanism (27). We tested GnRH and its agonist analog peptides (except GnRH-OH) for their ionophoretic abilities using LUVs of DMPC containing the Ca^{2+}-sensitive dye Arsenazo III trapped within the vesicles. Only the superagonist peptide des-Gly^{10}[D- Trp^6,Pro^9-NHEt]GnRH showed a significant degree of Ca^{2+} influx. The data obtained are depicted in Fig. 4, which shows the time-dependent entry of Ca^{2+} into Arsenazo III-laden bilayer vesicles in the presence of this peptide. A part (<20%) of the ion transport, however, was determined to be caused by leakage of the dye as judged by the effect of the addition of EDTA at the end of the experiment (Fig. 4; see below). An

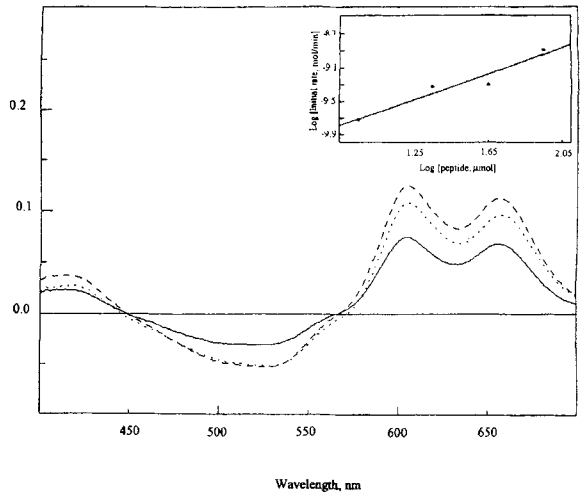

Figure 4. Peptide-mediated translocation of Ca^{2+} into LUVs of DMPC (3.5 μmol) measured at 37 ± 1°C in 50 mM Hepes and 125 mM KCl, pH 7.4. The sample cuvette had LUV containing 3.6 mM Arsenazo III incubated with 3.5 mM external $CaCl_2$ and 30 μM [desGly^{10},D-Trp^6]GnRH. The reference cuvette had all the components except the peptide. Difference spectra shown were taken at 6 min (—), 15 min (---) and after addition of 5 mM EDTA (final concentration) at 16 min (...). Duration of spectral scan: 3 min.

estimate of the stoichiometry of the peptide/Ca^{2+} complex prevailing within the lipid bilayer can be obtained from the slope of the logarithmic plot of the rate of ion transport as a function of peptide concentration at fixed (excess) cation concentration (29; see Ref. 34 for treatment of transport kinetics). The slope of the best-fit line in the inset in Fig. 4 is close to unity, indicating a first-order interaction between the hormone and Ca^{2+}. This shows that a 1:1 peptide/Ca^{2+} complex in the lipid milieu is the species responsible for ion translocation as observed earlier by us in the insulin B chain (29). As in the studies on other hormones (29, 32, 33), tests for possible membrane disruption and membrane fusion by des-Gly^{10}[D-Trp^6,Pro^9-NHEt]GnRH under the conditions used in the transport experiments were made by the standard procedures (30). These tests, shown in Fig. 5, indicated that the peptide caused a relatively small extent of leakage of contents (~10%) and membrane fusion (~15%). In contrast, [Ala^6]GnRH, which showed a small but significant Ca^{2+} influx in the Arsenazo III experiment (data not shown), caused considerable leakage of contents and membrane fusion (~30%; Fig. 5) which almost fully accounted for its observed extent of Ca^{2+} translocation.

Discussion

Earlier studies on the structure-activity relation in GnRH have focused mainly on the primary structural requirement

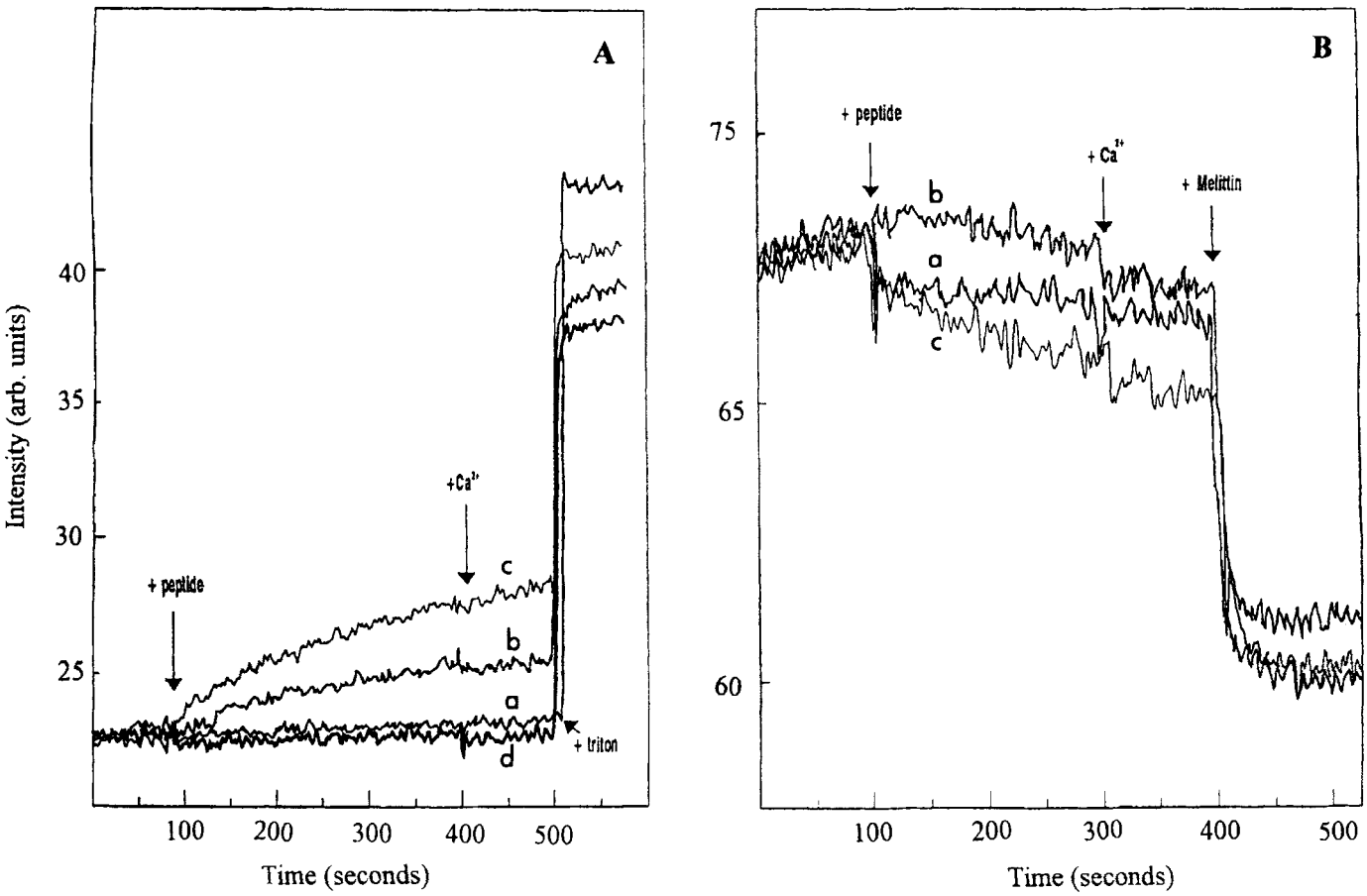

Figure 5. Leakage and fusion assays of GnRH peptides using DMPC LUV (3.5 μmol lipid). (**A**) Leakage assay. DMPC vesicles containing 12.5 mM ANTS and 45 mM DPX in 5 mM Hepes and 125 mM KCl, pH 7.4, were preincubated at 37°C. Peptides, Ca^{2+} and 10% (v/v) Triton X-100 were added at times shown by the arrows: a) 60 μM GnRH; b) 30 μM des-Gly^{10}[D-Trp^{6},Pro^{9}-NHEt]GnRH; c) 30 μM [Ala^{6}]GnRH; and, d) 1 μM A23187. Fluorescence excitation was at 384 nm and emission measured at 545 nm. Fluorescence increase caused by the addition of Triton X-100 was taken to represent 100% leakage. (**B**) Fusion assay. Two sets of DMPC vesicles one containing ANTS (25 mM in 5 mM Hepes and 125 mM KCl, pH 7.4) and the other containing DPX (90 mM in 5 mM Hepes and 125 mM KCl, pH 7.4) were mixed together and preincubated at 37°C . Peptides, Ca^{2+} and melittin (1 μM) were added at times indicated by the arrows. (**a**) 100 μM GnRH; (**b**) 30 μM des-Gly^{10}[D-Trp^{6},Pro^{9}-NHEt]GnRH; and (**c**) 30 μM [Ala^{6}]GnRH. Excitation was at 384 nm, and emission was monitored at 545 nm.

for agonist and antagonist activity (2–4). These indeed have been extremely beneficial in the synthesis of several medically useful GnRH analogs. In fact, GnRH is one of the few cases where superagonists more potent than the native hormone were discovered very early on (21) based on empirical amino acid replacement studies. Despite this accomplishment, an intrinsic knowledge of the *in vivo* structure of receptor-bound GnRH is still needed for understanding the hormone's action and for further progress in drug design. In the absence of knowledge of the three-dimensional structure of the GnRH receptor, the structure of the receptor-bound hormone can be sought, at best, only by modelling studies (11). As an alternative, we have tried here to understand this structure by examining the structures of free and Ca^{2+}-bound forms of GnRH agonists. With the help of Dr. Karten at the National Institutes of Health, we were able to obtain GnRH agonist peptides whose biological activities cover a wide range, from the inactive GnRH-OH to the superactive des-Gly^{10}[D-Trp^{6},Pro^{9}-NHEt]GnRH. The relative activities of the peptides, as determined by the luteinizing hormone-release assay (Karten, personal communication) are: GnRH, 1.0; GnRH-OH, 0.1; [Ala^{6}]GnRH, 0.04; [D-Ala^{6}]GnRH, 3.5–5.0; des-Gly^{10}[D-Ala^{6}]GnRH, 10; and, des-Gly^{10}[D-Trp^{6},Pro^{9}-NHEt]GnRH, 100–145. As pointed out by Karten and Rivier (4), the close agreement between the potencies of these analogs determined by *in vivo* and *in vitro* methods is attributable to the dominant role played by the conformation of these analogs rather than by differences in their assimilation in the body. This provided an additional impetus for our seeking the conformational differences responsible for their relative bioactivities. Structural studies on weak as well as strong agonists of

GnRH are essential (11) but not available as yet. In this study, we have examined the structures of a series of agonist analogs in the structure-promoting solvent TFE.

There was another rationale for this study. Experiments from our laboratory on insulin (29), substance P (32), glucagon (33) and oxytocin (35, 36), and from other laboratories on enkephalin (37), gastrin (38, 39) and cholecyokinin (39), have shown that many peptide hormones are capable of forming stable, stoichiometric complexes with Ca^{2+} in lipid-mimetic solvents and inside the lipid bilayer of synthetic liposomes where some of these hormones act as Ca^{2+} ionophores (29, 32, 33). The Ca^{2+}-bound hormone is expected (27) and seen (36) to be more rigid than the free hormone with polar peptide carbonyls bound to the cation in the interior and hydrophobic residues exposed to the solvent. These attributes make the Ca^{2+}-bound hormone more likely to represent the receptor-bound species than the flexible, free hormone. Earlier observations from our laboratory that support this possibility are: a) the so-called message regions, which are involved in receptor activation (40), are the main sites of Ca^{2+} binding in many hormones (41); b) differences in the bioactivities of structurally similar hormones such as oxytocin and vasopressin can be related to their drastically different Ca^{2+}-binding behaviors (35); and c) the close structural resemblance between the alkaloid ligand morphine and the Ca^{2+} bound, but not the free, forms of several agonists of the μ-opioid receptor (42). In this context, structural studies on weak as well as strong agonists of a given hormone, such as the present study on GnRH, would help to verify further the role of Ca^{2+} in dictating the bioactive conformation of peptide hormones by way of seeking the correlation, if any, between their bioactivities of these analogs on the one hand and their Ca^{2+}-binding properties on the other.

The Ca^{2+}-binding experiments were conducted in TFE, a solvent closely mimicking the relatively nonpolar environment of the biological membrane and used as such in other studies (43). Such an environment provides short linear peptides an opportunity to form intramolecular hydrogen bonds as well as interact with metal ions via the peptide carbonyl groups. [As noted by Donzel *et al.* (44) in their study of charge-transfer complexes in GnRH, in addition to their biological relevance, structural studies on peptide hormones under different conditions provide an insight into the forces determining the conformations and interactions of peptides in a given environment.] Control experiments in water showed no significant Ca^{2+} binding to the GnRH peptides. This was expected on the basis of the relatively disordered structure of short peptides in water (31) and the strong hydration of the Ca^{2+} ion (45). In the presence of millimolar levels of extracellular Ca^{2+} and a binding affinity of ~50 μM for the ion, significant amounts of the Ca^{2+} complex of GnRH can be present in the lipid-like milieu. GnRH previously has been shown, by potentiometric titration, to bind Cu^{2+}, Ni^{2+} and Zn^{2+} in water (46) forming stable but weak complexes involving nitrogen donors. No conformational analysis of these complexes were made, however. In our study on TFE, GnRH and the analog peptides showed that selective binding of Ca^{2+} over Mg^{2+} did not bind the monovalent Na^+. The parallel between this observation and the biochemical finding on the requirement of Ca^{2+} but not Mg^{2+} and Na^+ for GnRH action (8) may be worth noting. The study of Ca^{2+} translocation across the lipid bilayer in a synthetic liposome mediated by the GnRH peptides showed that only des-Gly^{10}[D-Trp^6, Pro^9-NHEt]GnRH showed a significant extent of ion translocation. This may reflect on the increased lipophilicity of this peptide compared with the others. This, in turn, may have a bearing on the increased potency of this peptide. In this context, it is interesting that in 1977 Bradley *et al.* (47) had observed selective binding of Ca^{2+} over K^+ by linear hexapeptide analogs of elastin both in TFE and in water. These hexapeptides also mediated selective cation transport across an artificial lipid bilayer. The authors concluded that "the study of cation complexation in peptides and their reconstitution in artificial membranes should be helpful in understanding the relationship between ligand protein binding and biological efficacy."

In the absence of Ca^{2+}, the CD and fluorescence spectral features of GnRH and all of the agonist peptides do not show any major differences implying that their conformations are similar (Fig. 1) . However, marked differences exist in their interaction with Ca^{2+} in terms of binding stoichiometry as shown by the data in Figs. 2 and 3. Of particular interest is the nature of the spectral change (which reflects structural change) for each peptide at different Ca^{2+} concentrations. The native hormone and the superagonists show an indication of a biphasic binding of the cation at two different sites in the molecule (Figs. 2 and 3), whereas the weaker agonists show a single binding site. (Compare the biphasic Ca^{2+} binding by substance P and single-site binding by its 7–11 fragment in Ref. 33.) A clear separation between the binding of the first and second Ca^{2+} to GnRH also was observed in our preliminary NMR spectral data on the hormone (Mazza, V. and Ananthanarayanan, V.S., unpublished data). The chemical shift change *versus* Ca^{2+}concentration plots for some of the NH protons showed that binding the first Ca^{2+} ions causes significantly more perturbation of the middle and C-terminal residues (e.g. Gly^6, Leu^7, Arg^8 and Gly^{10}) than the N-terminal residues (e.g. Trp^2).

A conformational interpretation of the differences in the Ca^{2+}-binding isotherms of each of the GnRH peptides should await the NMR determination of the three-dimensional structures of their free and Ca^{2+}-bound forms, as done for oxytocin (36). Based on the existing data, it appears that the β-turn in middle region of the peptide (11) may be responsible for the binding of the second Ca^{2+} ion in GnRH. This conformation may be more amenable for Ca^{2+} binding in GnRH and the superagonists than in the weak agonists. Although additional experimental data admittedly are necessary to verify this, the remarkably well-distinguished Ca^{2+} binding curves for the agonist peptides (which parallel their bioactivities in terms of their appearance and relative displacement) cannot be coincidental and warrant further considerations about the role of Ca^{2+} in the bioactive conformation of GnRH. It is likely that the reason for the requirement of extracellular Ca^{2+} for GnRH action may lie partly in the need for the Ca^{2+}- bound hormone rather than the free hormone for productive interaction with the receptor. Such a possibility has been considered in other cases (48) and is worth pursuing further.

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