INTRINSIC TRYPTOPHAN FLUORESCENCE OF MEMBRANES OF GH_3 PITUITARY CELLS: QUENCHING BY THYROTROPIN-RELEASING HORMONE*

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Summary: The intrinsic tryptophan fluorescence of membranes prepared from the GH_3 strain of hormone-producing pituitary cells was monitored by spectrofluorometry. Membranes of GH_3 cells have specific receptors which bind thyrotropin-releasing hormone (TRH). When TRH binds to GH_3 membranes there is quenching of tryptophan fluorescence. The kinetics of the change in fluorescence of GH_3 membranes and of TRH binding are similar. In addition, the concentration of TRH required to produce a half-maximum change in fluorescence is 10 nM, and that required for half-maximum binding of TRH to receptors is 11 nM. Inactive TRH analogs which do not bind to TRH receptors likewise do not alter GH_3 membrane fluorescence, and a pituitary cell strain which lacks TRH receptors does not change membrane fluorescence on incubation with TRH. We conclude that the TRH-receptor interaction in GH_3 membranes is associated with a change in membrane conformation that is readily measured by differential spectrofluorometry.

Introduction

The GH_3 strain of rat pituitary cells synthesizes and secretes prolactin and growth hormone (1). GH_3 cells in culture respond to the hypothalamic tripeptide, thyrotropin-releasing hormone (TRH), L-pGlu-L-His-L-ProNH_2. Within a period of less than one hour, TRH stimulates the release into the medium of stored intracellular prolactin (2). After a lag of about 4 hours, TRH also increases the rate of prolactin synthesis and decreases growth hormone production (3-5). These effects on hormone synthesis become maximum after 24-48 hours of treatment. The effects of TRH on GH_3 cells appear to be mediated by specific membrane receptors for the tripeptide. TRH binds only to cells which respond biologically to the peptide (6). Binding occurs rapidly, precedes the biological responses, and is initially reversible (6). The binding sites are of limited capacity; half-saturation

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of TRH receptors occurs at a concentration of 11 nM, a value which approximates the concentration required for half-maximum effects on hormone synthesis, 2 nM (5, 6). In addition, recent studies have shown that TRH regulates the number of its own receptors in GH_3 cells (7). The binding characteristics of TRH to GH_3 cellular receptors are similar in both intact cells and a particulate membrane fraction prepared from broken cells (6).

The purpose of the present study was to determine whether the interaction between TRH and its receptors resulted in a measurable alteration in the conformation of the GH_3 cell membrane. An excellent probe for such conformational changes is differential spectrofluorometry which was introduced by Fasman and his colleagues (8, 9). The results of such measurements reveal changes in tryptophan fluorescence which correlate well with the binding characteristics of TRH to its receptor.

Methods and Materials

GH_3 cells. Methods of culture of GH_3 and GH_12C_1 cells have been described previously (10). GH_12C_1 cells produce growth hormone but not prolactin, they lack TRH receptors, and they do not respond biologically to TRH (6). For the experiments described in this report, GH_3 cells were grown in either monolayer (10) or suspension culture (11), and GH_12C_1 cells were grown in monolayer. Cells were harvested from monolayer by scraping with a rubber policeman, and from suspension by centrifugation.

Preparation of membrane fraction. Harvested intact cells (1 to 10×10^7 cells) were washed twice with 0.15 M NaCl- 0.01 M sodium phosphate buffer, pH 7.5. The cells were then suspended in 3 to 5 ml of ice cold Tris-Mg buffer (20 mM Tris-HCl, 2 mM $MgCl_2$, pH 7.6). After 5 minutes at 0°C, the cells were ruptured by 25 strokes in a Dounce homogenizer (pestle A) and centrifuged for 10 minutes at 4000xg. The pellet was suspended in Tris-Mg buffer and the concentration of the membrane suspension was determined by the absorbance at 280 nm measured with a Cary 14 recording spectrophotometer, in a 1 cm quartz cell. The membrane preparations were diluted to a final A_{280} of approximately 0.20 prior to use.

Measurement of fluorescence. Fluorescence measurements were made on a newly designed and constructed differential fluorometer (12).

The instrument time-shares two fluorescent signals using one light source, one photomultiplier and two monochromators. The excitation signal is chopped by a rotating mirror which in addition to having two reflecting sectors, has a dark reference as well. The signals are recombined by a

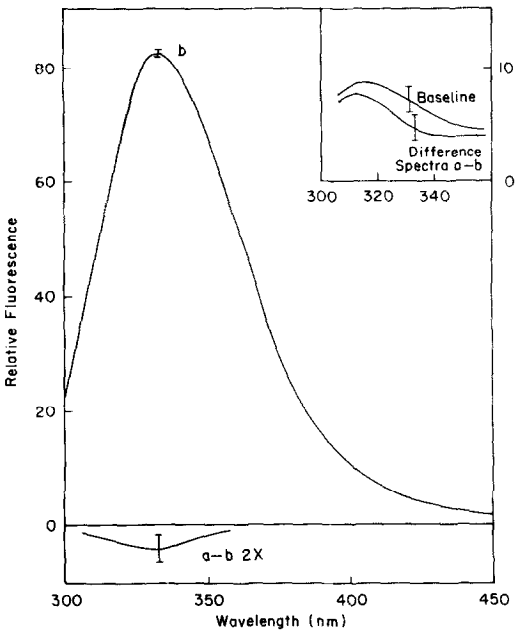

Figure 1. Fluorescence spectra of GH_3 membranes with and without TRH. Suspensions of GH_3 membranes were prepared for scanning as described under Methods. Curve a (not shown): absolute emission spectra of GH_3 membranes with 99 nM TRH versus buffer. Curve b: absolute emission spectrum of GH_3 membranes versus buffer. Curve a-b: difference spectrum between GH_3 membranes with and without 99 nM TRH, corrected for baseline deviation. Membrane suspensions were incubated with or without TRH for 20 min at 0°C before scanning. The baseline value (a-b) was obtained with two identical cells containing buffer solutions in the two compartments. This curve was subtracted from all differential fluorescence measurements in order to obtain the true difference. The insert shows an observed baseline for a difference spectrum and an uncorrected difference spectrum. The vertical bars give the instrumental noise error. Instrumental conditions: photomultiplier tube voltage, 780 volts; sensitivity, 1×10^{-8} amp; excitation slit width, 3.0 mm; emission slit width, 2.7 mm; excitation wavelength, 285 nm; full scale of Y range in recorder, 1 volt/div. for absolute spectrum and 0.5 volts/div. for difference spectrum; scan time, 5 min./100 nm; temperature of cell chambers, 10°C; cell path length, 1 cm.

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second rotating mirror which directs these alternate fluorescent signals into a monochromator.

The output of the photomultiplier is a 13 Hz square wave with the dark reference between each signal. These waveforms are synchronously detected by two lock-in amplifiers. A differential amplifier produces a signal equal to the difference of the two fluorescent samples. In addition, a ratiometer takes the ratio of the two fluorescent spectra when needed.

Differential spectrophotometry was performed as follows. Three

ml of a suspension of GH_3 or $GH_1 2C_1$ cell membranes in Tris-Mg buffer were placed in each of two quartz cuvettes. An aliquot of the appropriate stock solution of TRH or TRH analog, in Tris-Mg buffer, was added to one cuvette in a volume of 5-50 μl, and an equal volume of buffer was added to the other cuvette. The cuvettes were then incubated in an ice-water mixture for 20 min. unless otherwise noted. Immediately before scanning the quartz cells were inverted.

Materials. Synthetic TRH and peptide analogs were the gift of Abbott Laboratories, N. Chicago, Illinois.

Results

The fluorescence spectrum, corrected for baseline deviations, of GH_3 membranes alone is shown in Fig. 1, curve b, with the peak at 333 nm. This fluorescence emission wavelength is that associated with tryptophan (13). As no shoulder is visible in the 303 nm region, we assume that there is negligible tyrosine fluorescence (8). On the addition of TRH (99 nM), the difference spectrum (labeled a-b) was obtained, run at twice the sensitivity of that of curve b. The insert in Fig. 1 shows the actual baseline and the difference spectrum before subtracting the baseline. Vertical bars on the curves indicate the relative noise levels. The actual plots obtained from the instrument are given in Fig. 2. The spectrum of GH_3 membranes alone (plus the baseline) is shown in Fig. 2a, and the difference spectrum of GH_3 membrances with and without TRH is shown in Fig. 2b [baseline (upper curve) and difference spectrum (lower curve)].

A study was made to determine the optimal incubation time with TRH (99 nM, at 0°) for the production of the maximum difference fluorescence (δ F). These results are shown in Fig. 3. It is seen that an incubation time of approximately 20 minutes yields the maximum δ F.

The dependence of δ F of GH_3 membranes on the concentration of TRH is shown in Fig. 4. The maximum δF is obtained with 100 nM TRH and the half-maximum δF is obtained at approximately 10 nM TRH, a value which corresponds closely to the concentration of TRH which half-saturates TRH receptors, 11 nM (5, 6).

To examine the specificity of the effects of TRH on the fluorescence of membranes from GH_3 cells we performed two kinds of control experiments. Analogs of TRH (5) which have little or no biological activity and which do not bind significantly to TRH receptors on GH_3 cells were tested for their effects on the fluorescence of GH_3 membranes. As shown in

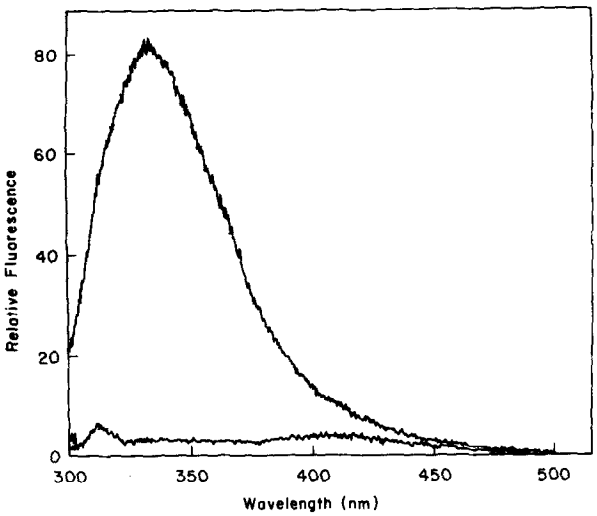

Figure 2(a). Instrument plot of the fluorescence spectra of GH_3 membranes, upper curve. Baseline, lower curve. Conditions as in Fig. 1 (curve b).

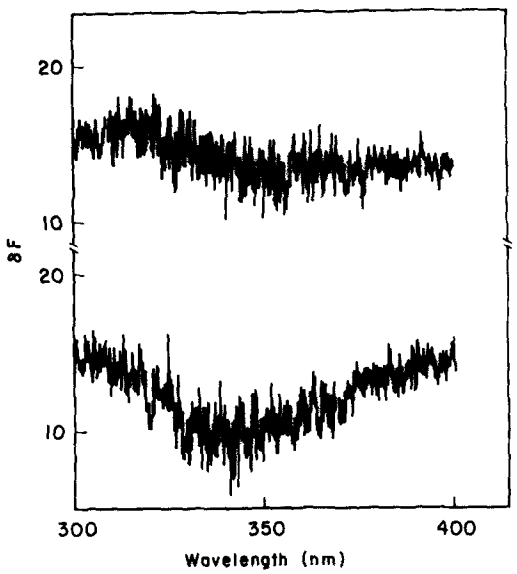

Figure 2(b). Instrument plot of the difference spectrum between GH_3 membranes with (99nM) and without TRH. Baseline, upper curve; lower curve difference spectrum. Conditions as in Fig. 1 (curve a-b).

Table 1, four such analogs of TRH had little or no effect on the fluorescence of GH_3 membranes. For the second type of control, we examined the effect of TRH itself on membranes prepared from $GH_1 2C_1$ cells, a strain of pituitary cells that lacks specific receptors for TRH (6). The results given in Table 2 show that TRH had no significant effect on the fluorescence of $GH_1 2C_1$ membranes.

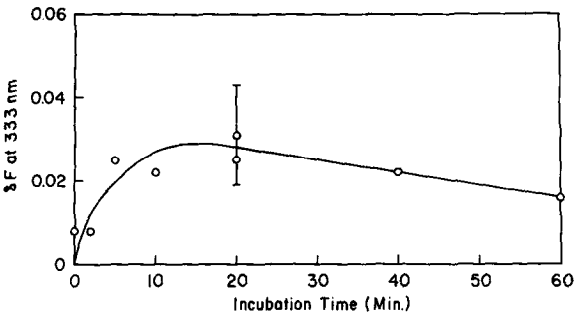

Figure 3. Effect of time of incubation with TRH on fluorescence of GH_3 membranes. Suspensions of GH_3 membranes were incubated at 0°C with or without 99 nM TRH for the indicated times before scanning. Difference fluorescence (δF) was then measured for GH_3 membranes with and without TRH as described in Fig. 1. A fresh aliquot of GH_3 membranes was used for each experimental point. δF was measured at 333 nM and was calculated as follows: $\delta F = \frac{(\text{Rel F, a-b})_{333}}{(\text{Rel F, b})_{333}}$ [1] The vertical bars give the instrumental noise error.

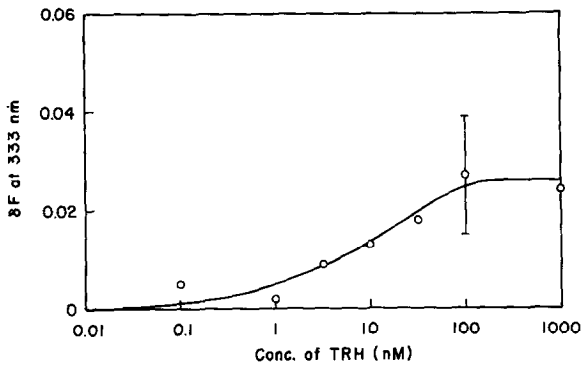

Figure 4. Effect of TRH concentration on fluorescence of GH_3 membranes. Difference fluorescence (δF) of GH_3 membranes with or without the indicated concentrations of TRH was measured as described in Fig. 1. Membranes were incubated with or without TRH for 20 min at 0°C before scanning. Fresh cell suspensions were used for each experimental point, and various TRH concentrations were tested in random order. δF at 333 nM was calculated according to eq. [1] in Fig. 3. The vertical bars give the instrumental noise error.

Discussion

Fluorescence spectroscopy has been widely used to detect changes in the environment (e. g. solvent perturbation, substrate binding, conformational changes) about tyrosine or tryptophan residues in a protein molecule (13). In addition to these causes of fluorescence alteration, specific quenching effects induced by environmental factors can cause large

TABLE 1

EFFECTS OF TRH AND TRH ANALOGS ON FLUORESCENCE OF GH_3 MEMBRANES

Peptide	Conc. (nM)	δF at 333 nm
pGlu-His-ProNH_2 (TRH)	9.8	0.015 ± 0.011
	99.0	0.029 ± 0.012
pGlu-His-Pro	103	0.002 ± 0.012
	1020	-0.001 ± 0.012
pGlu-His-Pro$N(CH_3)_2$	99.0	0.009 ± 0.014
	980	0.006 ± 0.014
pGlu-His-NHEt	103	0.004 ± 0.014
	1020	0.004 ± 0.014
pGlu-His-AlaNH_2	103	0.003 ± 0.013
	1020	0.016 ± 0.014

GH_3 membranes (A_{280}= 0.21) were incubated for 20 min at 0°C with TRH or TRH analogs and δF monitored and calculated as described in Fig. legends 1 and 3, respectively. The values following the ± signs give the instrumental noise error. Most determinations were performed in duplicate and the average δF is given.

fluorescence changes (8). The application of differential spectrophotometry has greatly increased the sensitivity of this approach (8, 9).

Tryptophan fluorescence in aqueous solution displays an emission peak near 340 nm on excitation at 285 nm. However, on lowering the polarity of the environment an increase in fluorescence and a corresponding blue shift in the fluorescence is observed (9, 13). Thus, the peak emission observed at 333 nm for the GH_3 membrane preparation is due to Try and would imply that the Try residue (or residues) responsible for the observed fluorescence is not totally exposed to the aqueous media but is either partially buried in the matrix of the membrane or in a hydrophobic environment. On the binding of TRH to GH_3 membranes there is a small but significant and highly reproducible decrease in fluorescence (δ F ~ 0.03) is observed (Figs. 1, 2). This quenching of fluorescence could be caused by (a) a conformational change in the membrane whereby a specific quenching group, e. g. COO^- (14) is brought closer to the Try residue, (b) the binding of TRH might occur near or on Try residues allowing for energy trans-

TABLE 2

LACK OF EFFECT OF TRH ON FLUORESCENCE OF $GH_1 2C_1$ MEMBRANES

Conc. of TRH (nM)	δ F at 333 nm
9.9	0.001 ± 0.013
99.0	0.003 ± 0.013
980	0.005 ± 0.014

$GH_1 2C_1$ membranes (A_{280}=0.24) were incubated for 20 min. at 0°C with TRH and δF was monitored and calculated as described in Fig. legends 1 and 3, respectively. The values following the ± signs give the instrumental noise error.

fer, or (c) by direct quenching. As the peak emission is not shifted, the environment surrounding the fluorescent Try residues is not greatly altered in polarity on binding of TRH. Unless Try residues are involved in an important way in the TRH receptor itself, the δ F which we measure in response to the binding of TRH to GH_3 membranes implies that a rather large change in membrane conformation occurs on binding the small ligand, TRH, a tripeptide. At saturation, one molecule of TRH is bound to membranes which contain approximately 10,000 molecules of Try. Thus, it is most unlikely that the observed change in fluorescence is due to quenching of a single or very small number of Try residues on binding of TRH. Such changes in membrane conformation must now be considered in evaluating the mechanisms of TRH action on prolactin and thyrotropin release and synthesis (2, 15).

Biologically inactive TRH analogs, which do not bind to TRH receptors (5), do not alter the fluorescence of GH_3 membranes (Table 1). Furthermore, $GH_1 2C_1$ cells, which lack TRH receptors and do not respond biologically to TRH, show no change in fluorescence in response to TRH (Table 2). These results imply that specific TRH receptors are required for the observed changes in membrane fluorescence (Fig. 1 and 2). In addition, the kinetics of TRH binding to receptors (6) is similar to the kinetics of the effect of TRH on fluorescence shown in Fig. 3. Finally, the concentration dependence of TRH binding to GH_3 receptors, which is half-maximum at 11 nM TRH (5, 6), corresponds well to the concentration of 10 nM required to produce half-maximum δF (Fig. 4).

It should be emphasized that our measurements reflect the intrinsic fluorescence of Try residues in the membrane fraction and that fluorescent probes, which may themselves alter membrane conformation, were not used in these experiments. Furthermore, our results demonstrate the general utility of differential spectrofluorometry (8, 9) and add to several previous studies on membranes utilizing fluorescence (16-18) as well as measuring fluorescence changes in response to a hormone (17, 18).

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