

# Direct visualization of receptors for thyrotropin-releasing hormone with a fluorescein-labeled analog

(pituitary/GH<sub>4</sub>C<sub>1</sub> cells/prolactin/fluorescein isothiocyanate/thyroliberin)

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**ABSTRACT** Thyrotropin-releasing hormone (TRH) binds to specific receptors on GH<sub>4</sub>C<sub>1</sub> pituitary tumor cells. A fluorescently labeled analog of TRH was synthesized by coupling pGlu-His-ProNH(CH<sub>2</sub>)<sub>6</sub>NH<sub>2</sub> to fluorescein isothiocyanate. The fluorescein-labeled peptide (FL-TRH) stimulated prolactin synthesis and release by GH<sub>4</sub>C<sub>1</sub> cultures and bound to TRH receptors with an apparent K<sub>d</sub> of 400 nM. Binding of FL-TRH to unfixed, viable GH<sub>4</sub>C<sub>1</sub> cells was followed by fluorescence microscopy. After incubation with 1.4 μM FL-TRH for 1 hr at 37°C, the surface of all cells was fluorescent and patches of intense fluorescence were evident. Control cultures incubated with FL-TRH and excess TRH were not fluorescent, and a line of pituitary tumor cells which lacks TRH receptors displayed little fluorescence after incubation with FL-TRH. When GH<sub>4</sub>C<sub>1</sub> cells were incubated with FL-TRH for 1 hr at 37°C and then with excess TRH for an additional 1 hr, the fluorescence associated with the cells was diminished to control levels. The results demonstrate that the fluorescein-labeled peptide labels specific TRH receptors.

The tripeptide thyrotropin-releasing hormone (TRH; thyroliberin), pGlu-His-ProNH<sub>2</sub>, is secreted from the hypothalamus into the hypothalamic-pituitary portal system. At the anterior pituitary, TRH simulates the release of both thyrotropin and prolactin (1-3). In GH<sub>4</sub>C<sub>1</sub> cells, a clonal line of rat pituitary tumor cells, TRH increases the synthesis and release of prolactin (4). The effects of TRH on normal pituitaries and GH<sub>4</sub>C<sub>1</sub> cells are thought to be initiated by association of TRH with a specific cell surface receptor, which has been identified in both systems (5-7). [<sup>3</sup>H]TRH binds rapidly to receptors on GH<sub>4</sub>C<sub>1</sub> cells with noncooperative kinetics; the apparent K<sub>d</sub> is 10 nM and the average number of sites per cell is approximately 100,000 (6, 8). There is a strong correlation between the biological activity of TRH analogs (measured as ability to stimulate prolactin synthesis) and the affinity for TRH receptors (9).

Peptide hormones are generally thought to bind initially to receptors on the plasma membrane, but the location of TRH receptors is uncertain. In broken cell preparations [<sup>3</sup>H]TRH binds to fractions containing plasma membranes but also to nuclei and smooth microsomes (10). There is little or no binding to soluble components (11). Isolated cell nuclei have also been reported to bind [<sup>3</sup>H]TRH (12). In order to investigate early events in TRH action, we have synthesized a fluorescein-labeled derivative of TRH (FL-TRH) and used it to visualize binding to TRH receptors in living GH<sub>4</sub>C<sub>1</sub> cells.

## MATERIALS AND METHODS

Unlabeled TRH and pGlu-His-ProNH(CH<sub>2</sub>)<sub>6</sub>NH<sub>2</sub> were generous gifts from Abbott. [<sup>3</sup>H]TRH (100 Ci/mmol; 1 Ci = 3.7 × 10<sup>10</sup> becquerels) was purchased from New England Nuclear,

and fluorescein isothiocyanate (isomer I) was from Sigma. Tissue culture media and normal sera were from GIBCO, and thyrotropin-deficient serum was obtained from a surgically thyroidectomized young steer.

Pituitary cell lines were grown as described (13) in monolayer culture in Ham's F10 medium supplemented with 15% horse serum and 2.5% fetal calf serum (unless noted otherwise), in a humidified atmosphere of 5% CO<sub>2</sub>/95% air. GH<sub>4</sub>C<sub>1</sub> cells were obtained from Armen H. Tashjian, Jr. (Harvard School of Public Health, Boston). GH-Y cells arose spontaneously from the GH<sub>4</sub>C<sub>1</sub> line in the laboratory of Priscilla S. Dannies (Yale University Medical School, New Haven, CT). We have carried the GH-Y line since 1978 and have not detected any increase in TRH receptor levels or TRH responsiveness. The concentrations of prolactin in culture media and cells were determined by double-antibody radioimmunoassay using preparations supplied by Albert Parlow (Hormone Distribution Program, National Institute of Arthritis, Metabolism, and Digestive Diseases). Specific binding of [<sup>3</sup>H]TRH was determined in cell monolayers or broken cell preparations as described (6). Nonspecific binding was determined in control reactions containing excess unlabeled TRH; nonspecific binding was less than 5% of total binding to GH<sub>4</sub>C<sub>1</sub> cells and has been subtracted.

For use in microscopy, cells were grown on glass cover slips. The cover slips were rinsed in serum-free Ham's F10 medium and then incubated in serum-free medium containing FL-TRH and TRH, as indicated in the text, at 37°C for 1-2 hr. The cover slips were gently rinsed in ice-cold 0.15 M NaCl, inverted onto slides, and viewed immediately in a Zeiss Photomicroscope III with epifluorescence. In all experiments the exposure conditions, development, and printing were identical for control and experimental cells.

## RESULTS

**Synthesis of a Fluorescent Analog.** FL-TRH was synthesized by coupling pGlu-His-ProNH(CH<sub>2</sub>)<sub>6</sub>NH<sub>2</sub> to fluorescein isothiocyanate by standard procedures (14). [The peptide used for coupling is active in stimulating the release and synthesis of prolactin by pituitary tumor cells (15).] One milligram of pGlu-His-ProNH(CH<sub>2</sub>)<sub>6</sub>NH<sub>2</sub> was treated with 5 mg of fluorescein isothiocyanate for 18 hr at 4°C in NaHCO<sub>3</sub>/Na<sub>2</sub>CO<sub>3</sub> buffer at pH 7.0. The coupled product was separated from unreacted starting materials by filtration through Sephadex G-10 followed by chromatography on DEAE-cellulose. FL-TRH did not appear to be contaminated with any Pauly-positive or fluorescent material as judged by thin-layer chromatography in several systems. The overall yield was 80%. The concentration of FL-TRH was determined spectrophotometrically at 495 nm using  $\epsilon = 7.4 \times 10^4$

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Abbreviations: TRH, thyrotropin-releasing hormone (thyroliberin); FL-TRH, fluorescein-labeled analog of thyrotropin-releasing hormone.

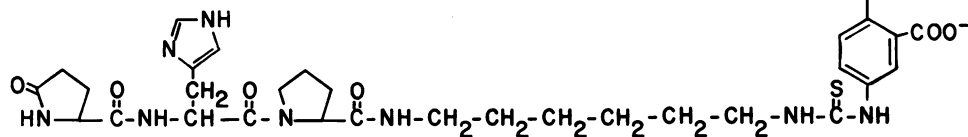


FIG. 1. Structure of FL-TRH.

$M^{-1} \text{ cm}^{-1}$ . The structure of FL-TRH is shown in Fig. 1; the fluorescein portion of the molecule is separated from the biologically active pGlu-His-Pro-moiety by a six-carbon spacer arm.

**Activity of FL-TRH.** The biological activity of FL-TRH was demonstrated by its ability to cause a 3-fold increase in the rate of prolactin synthesis by  $\text{GH}_4\text{C}_1$  cells (Fig. 2A) and to stimulate the release of stored prolactin (Table 1). The effect of FL-TRH on prolactin release was measured in a 1-hr incubation under conditions identical to those used in microscopy. The affinity of FL-TRH for TRH receptors was determined from its ability to compete with  $[^3\text{H}]\text{TRH}$  for binding to receptors on membrane fractions at  $0^\circ\text{C}$  and on intact cells at  $37^\circ\text{C}$  (Fig. 2B). FL-TRH exhibited an apparent  $K_d$  of 300–400 nM, so the peptide is about 2.5% as active as TRH. FL-TRH is at least 50% as potent as the

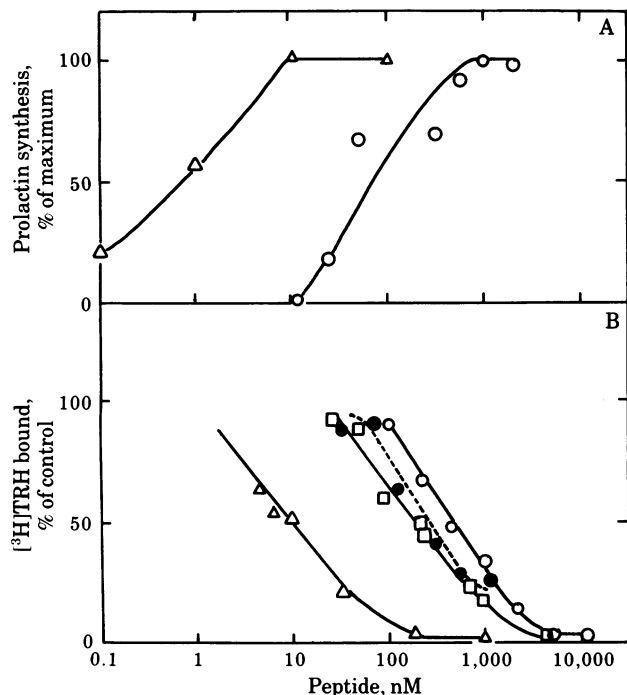


FIG. 2. Biological activity of FL-TRH. (A) Ability of FL-TRH to stimulate prolactin synthesis. Replicate 35-mm dishes of  $\text{GH}_4\text{C}_1$  cells were incubated for 72 hr with FL-TRH ( $\circ$ ) or TRH ( $\Delta$ ). The amount of prolactin secreted into the medium was measured by radioimmunoassay. The average of duplicate determinations is shown. (B) Affinity of FL-TRH for TRH receptors. Crude membrane fractions from  $\text{GH}_4\text{C}_1$  cells were incubated with 10 nM  $[^3\text{H}]\text{TRH}$  and unlabeled TRH ( $\Delta$ ), pGlu-His-ProNH( $\text{CH}_2$ ) $_6$ NH $_2$  ( $\square$ ), or FL-TRH ( $\circ$ ) for 1 hr at  $0^\circ\text{C}$ ; then, bound  $[^3\text{H}]\text{TRH}$  was determined by a nitrocellulose filter assay (6). Replicate monolayer cultures of  $\text{GH}_4\text{C}_1$  cells were incubated with 10 nM  $[^3\text{H}]\text{TRH}$  and various concentrations of FL-TRH for 1 hr at  $37^\circ\text{C}$  before determination of specifically bound  $[^3\text{H}]\text{TRH}$  ( $\bullet$ ) (6). The apparent dissociation constants of the peptides were determined by comparing the concentrations necessary for 50% displacement with that for unlabeled TRH ( $K_d = 10^{-8}$  M for TRH).

analog used for coupling, which had an apparent  $K_d$  of 200 nM, so that minor contamination with the starting peptide would not interfere with FL-TRH binding.

**Visualization of TRH Receptors with FL-TRH.** In order to visualize TRH binding sites on living cells, monolayer cultures of  $\text{GH}_4\text{C}_1$  cells were incubated with  $1.4 \mu\text{M}$  FL-TRH at  $37^\circ\text{C}$  for 1 hr. FL-TRH occupied approximately 80% of available receptors under these conditions. The fluorescent peptide bound to the cells, yielding a dim but reproducible pattern of fluorescence (Fig. 3 A and B). All of the cells were fluorescent. The entire surface of the cells was fluorescent, but fluorescence was more intense at the periphery of the cells than over the nucleus. Small areas of aggregated, intense fluorescence were also evident. In contrast, when cultures were incubated with uncoupled fluorescein isothiocyanate, a diffuse, uniform fluorescence was observed (data not shown). Extremely bright fluorescence was seen over areas of dead cells and debris, as can be seen by comparing the fluorescent and brightfield pictures.

We performed two control experiments to demonstrate the specificity of FL-TRH binding. Nonspecific binding was measured by incubating  $\text{GH}_4\text{C}_1$  cells with  $1.4 \mu\text{M}$  FL-TRH and excess TRH (Fig. 3 C and D). The cells were clearly much less fluorescent than parallel cultures incubated with FL-TRH alone. In addition, the fluorescence that could be seen did not have the distinctive pattern associated with specifically bound FL-TRH. In a second control, a pituitary line lacking TRH receptors, GH-Y cells, was incubated with  $1.4 \mu\text{M}$  FL-TRH. GH-Y cells arose spontaneously from the  $\text{GH}_4\text{C}_1$  cells; they bound only 2% as much  $[^3\text{H}]\text{TRH}$  as the parent line, and prolactin synthesis was not stimulated by TRH (Table 2). Little fluorescence was associated with GH-Y cells after incubation with FL-TRH (Fig. 3 E and F).

To determine if the binding of FL-TRH was reversible, cells were incubated with  $1.4 \mu\text{M}$  FL-TRH for 1 hr at  $37^\circ\text{C}$  and then incubated an additional hour in the presence of  $100 \mu\text{M}$  TRH. The fluorescence associated with the cells under these conditions was decreased to a level resembling that seen for nonspecifically bound FL-TRH (Fig. 4). Longer incubations with excess TRH did not noticeably decrease the remaining fluorescence.

The effect of thyroid hormones on FL-TRH binding was determined by growing  $\text{GH}_4\text{C}_1$  cells for 72 hr in hypothyroid medium with or without addition of 50 nM L-triiodothyronine; the

Table 1. Stimulation of prolactin release by FL-TRH

Addition	Prolactin, ng/dish	
	Medium	Cells
None	1937 $\pm$ 136	2638 $\pm$ 310
TRH (1 $\mu\text{M}$ )	3820 $\pm$ 364	1692 $\pm$ 166
FL-TRH (2 $\mu\text{M}$ )	3584 $\pm$ 132	1490 $\pm$ 274

Replicate 35-mm culture dishes of  $\text{GH}_4\text{C}_1$  cells were rinsed once with medium and then incubated for 90 min at  $37^\circ\text{C}$  with 2 ml of medium modified as shown. The means  $\pm$  SEM of triplicate determinations are shown.

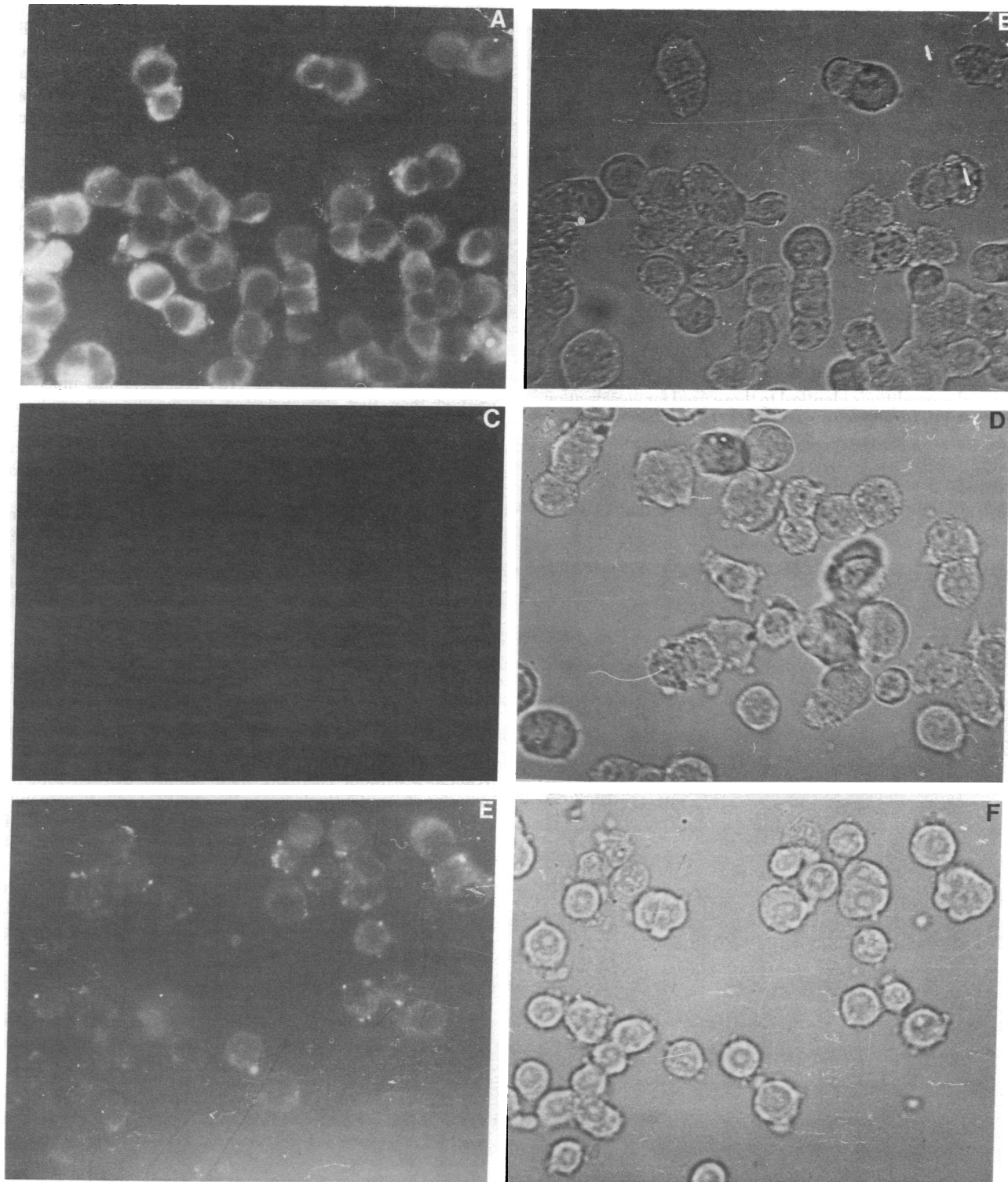


FIG. 3. Use of FL-TRH as a fluorescent probe for TRH receptors in viable  $\text{GH}_4\text{C}_1$  cells. Monolayers of  $\text{GH}_4\text{C}_1$  or  $\text{GH-Y}$  cells on glass coverslips were incubated with  $1.4 \mu\text{M}$  FL-TRH with or without TRH at  $37^\circ\text{C}$  for 1–2 hr. The coverslips were then gently rinsed in ice-cold  $0.15 \text{ M}$  NaCl, inverted onto slides, and viewed immediately by fluorescence (A, C, and E) and bright-field (B, D, and F) microscopy of the same fields. Exposure time was 3 min in all experiments with Kodak Tri-X film. ( $\times 500$ .) (A and B)  $\text{GH}_4\text{C}_1$  cells incubated with  $1.4 \mu\text{M}$  FL-TRH. (C and D)  $\text{GH}_4\text{C}_1$  cells incubated with  $1.4 \mu\text{M}$  FL-TRH and  $200 \mu\text{M}$  TRH. (E and F)  $\text{GH-Y}$  cells incubated with  $1.4 \mu\text{M}$  FL-TRH.

hypothyroid medium was Ham's F10 supplemented with 10% serum from a thyroidectomized calf (this serum contained undetectable levels of thyroxine and L-triiodothyronine). The cells were then incubated with FL-TRH and used for fluorescence microscopy as described in Fig. 3 A and B. In both sets of cultures, 100% of the cells were fluorescent.

## DISCUSSION

The results of this study show that specific binding of FL-TRH to TRH receptors can be observed by fluorescence microscopy on unfixed, viable cells. Biological responses to TRH (stimulation of prolactin release and uridine uptake) have been demon-

Table 2. Characteristics of pituitary cell lines

Cell line	$[^3\text{H}]\text{TRH}$ bound, fmol/mg cell protein	Growth hormone synthesis, $\mu\text{g}/\text{mg}$ cell protein/24 hr	Prolactin synthesis, $\mu\text{g}/\text{mg}$ cell protein/24 hr	
			Control	With TRH
$\text{GH}_4\text{C}_1$	$453 \pm 11$	$48.0 \pm 19.3$	$4.17 \pm 0.46$	$21.5 \pm 2.9$
$\text{GH-Y}$	$7 \pm 1$	$1.6 \pm 0.1$	$3.66 \pm 0.14$	$4.54 \pm 0.20$

Replicate 35-mm cultures of cells were incubated for 48 hr in Ham's F10 medium supplemented with 10% thyroidectomized calf serum, with or without  $1 \mu\text{M}$  TRH. The culture medium was collected for determination of hormone levels by radioimmunoassay. Dishes that had not received TRH were then used to measure  $[^3\text{H}]\text{TRH}$  bound after a 1-hr incubation with  $20 \text{ nM}$  radiolabeled hormone. Results are the means  $\pm$  SEM of triplicate determinations.

strated under conditions identical to those used for visualization of FL-TRH binding (15, 16) and FL-TRH-stimulated prolactin release. Because fluorescence appeared to be on the cell surface and FL-TRH was bound reversibly, the results support the view that TRH receptors are located on the plasma membrane. FL-TRH could be washed off the cells, indicating that it had not

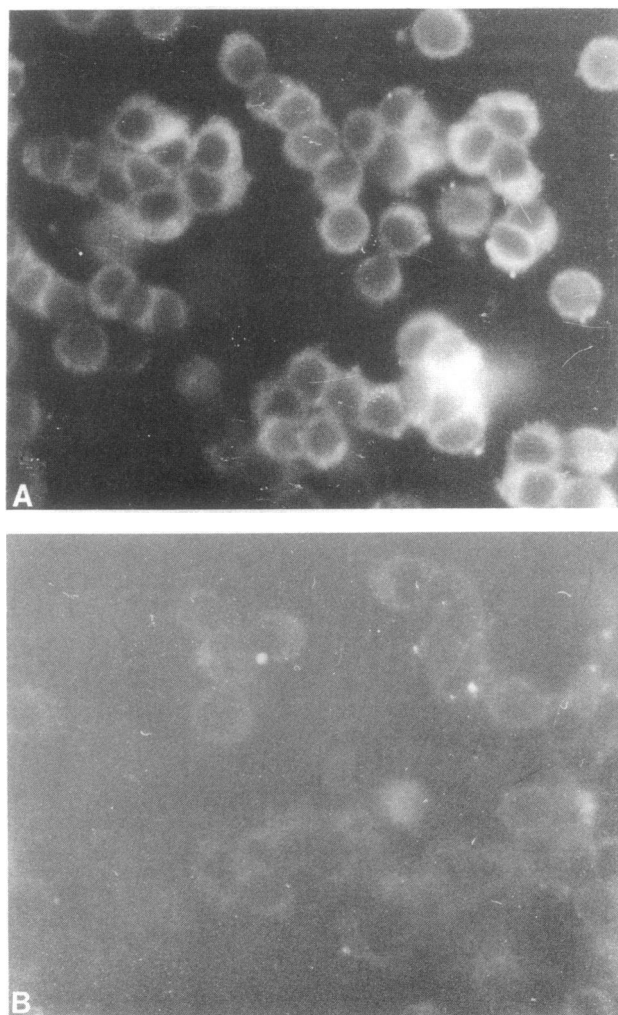


FIG. 4. Reversibility of FL-TRH binding. (A)  $\text{GH}_4\text{C}_1$  monolayers were incubated with  $1.4 \mu\text{M}$  FL-TRH for 1 hr at  $37^\circ\text{C}$ . (B) Some coverslips were then rinsed with saline and incubated an additional hour with  $100 \mu\text{M}$  TRH before viewing.

been irreversibly internalized after 1 hr at  $37^\circ\text{C}$ , a finding which is consistent with previous studies demonstrating that  $[^3\text{H}]\text{TRH}$  can dissociate from receptors on intact cells after several hours at  $37^\circ\text{C}$  (6, 8). Some peptide hormones that bind to cell surface receptors, such as insulin and epidermal growth factor, are rapidly internalized and degraded under conditions similar to those used in our experiments (17–19). In contrast, a fluorescently labeled opiate receptor agonist, rhodamine-enkephalin, was observed in clusters but did not internalize in several hours (20). The results with FL-TRH resemble those obtained with rhodamine-enkephalin. The TRH-receptor complex seems to internalize and become inactivated over 24–48 hr, as evidenced by the appearance of TRH metabolites in the cell (21) and “down-regulation” of TRH receptors (22) in this period.

$\text{GH}_4\text{C}_1$  cells were not uniformly fluorescent after incubation with FL-TRH, and the presence of patches of intense fluorescence suggests that some clustering of receptors occurred. However, the aggregation of TRH receptors was much less marked than that demonstrated for insulin and epidermal growth factor receptors (17–19). The areas of high fluorescent density may result from the movement of TRH receptors into aggregates after ligand binding or, alternatively, TRH receptors may exist in clusters before the peptide binds.

The cells used in these experiments were in logarithmic growth. Because all of the cells were fluorescent,  $\text{GH}_4\text{C}_1$  cells must bind TRH in all phases of the cell cycle. Faivre-Bauman *et al.* (23) have synchronized cultures of a closely related pituitary line and found that cells in S bind 40–80% more  $[^3\text{H}]\text{TRH}$  than do cells in  $\text{G}_1$  or  $\text{G}_2$ . There appeared to be differences in the fluorescence intensity of individual  $\text{GH}_4\text{C}_1$  cells (Figs. 2A and 4A) which may reflect such cell cycle-dependent differences in TRH binding capacity.

When pituitary tumor cells are incubated in hypothyroid medium, the addition of physiological concentrations of thyroid hormones causes a 50% decrease in the concentration of TRH receptors without changing their affinity (24). Thyroid hormones concomitantly decrease the ability of cells to respond to TRH. Previous studies with radioactive TRH could not distinguish whether thyroid hormones decreased the number of TRH receptors per cell or the fraction of cells with receptors. Because we found that 100% of cells were labeled with FL-TRH in cultures treated with L-triiodothyronine, it may be concluded that the average number of receptors per cell is regulated by thyroid hormones.

Fluorescent derivatives of TRH should be useful in identifying cells with TRH receptors, which is especially significant in light of the fact that TRH has been localized in various parts of the brain (25–27) and has been proposed to function as a neural transmitter as well as a pituitary releasing factor.

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