INTERACTION OF THYROTROPIN RELEASING FACTOR WITH MEMBRANE RECEPTORS OF PITUITARY CELLS.

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Received October 8, 1971

SUMMARY: Specific binding of ^{3}H-labelled TRF to mouse thyrotropic tumor membrane preparations and to intact pituitary cells is described. The binding is saturable with respect to ^{3}H-TRF under the conditions used and stoichiometrically competible by unlabelled TRF providing the basis for a workable *in vitro* assay for this releasing factor. Neither luteinizing hormone releasing factor (LRF) nor a biologically inactive dipeptide derivative of TRF compete for ^{3}H-TRF binding. The dissociation constant for ^{3}H-TRF binding to the tumor plasma membrane fraction is ca. 4×10^{-8}M at 0°C.

The site of action of thyrotropin releasing factor (TRF) (pGlu-His-Pro amide) at the level of the thyrotrophs is unknown(1). On the assumption that TRF is specifically recognized by the thyrotrophs, these cells should possess receptors with affinity for TRF. Recognition of the releasing factor may or may not be distinct from the terminal consequences of TRF action in the release of thyrotropin (TSH) but should be an integral part of the process. We wish to establish which molecules in the cell recognize TRF and to characterize the initial steps in the sequence of TRF action.

This report describes the direct measurement of TRF interaction with cell membranes using a nitrocellulose filter technique. Data are presented on the equilibrium constant, kinetics of the reaction and a competitive assay of unlabelled releasing factor.

Material and Methods:

Tritium labelled TRF was obtained from New England Nuclear Co.; the synthetic peptide was made *de novo* by a solid state method

developed in our laboratory (Monahan and Young, manuscript in preparation) utilizing ^{3}H-proline. The specific activity of the ^{3}H-TRF is 50 C/mmole.

The biological activity of the H-TRF was assayed in mice using the method described by Vale et al.(2). The specific biological activity (U/mg) of the ^{3}H-TRF is indistinguishable from that of synthetic unlabelled TRF. Synthetic TRF and TRF derivatives as well as synthetic luteinizing hormone releasing factor (LRF) were prepared in our laboratory(2,4).

TSH-secreting pituitary tumors used in these studies were induced in our laboratory by radio-thyroidectomy of LAF mice (Jackson Laboratories) with 200 μC of Na^{125}I after a 2-3 week period on an iodine-free diet; the tumors were maintained subcutaneously in radio-thyroidectomized animals. Thyrotropic tumors were homogenized and fractionated according to the procedures of Meldolesi, Jamieson and Palade(3). The cell fractions obtained were: nuclei (containing nuclei, mitochondria and zymogen granules), plasma membranes, endoplasmic reticulum membranes (both rough and smooth) and cytosol. Membrane concentrations were determined as dried weight.

The ^{3}H-TRF binding assay was carried out at 0°C, incubation time was 30 minutes except for kinetic experiments. All solutions were made up in a HEPES buffer (mM:NaCl, 137; KCl, 5; Na_2HPO_4, 0.7; N-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid (HEPES), 25; pH 7.2) with the exception that membrane preparations also contained 0.3M sucrose and 0.005M $MgCl_2$. Aliquots of membranes were mixed with various concentrations and specific activities of ^{3}H-TRF depending upon the experiment. Incubations were carried out in a final volume of 0.2 ml. Assay samples were diluted upon completion of the incubation with a 10-fold excess of ice cold saline and immediately filtered through a 25 mm Schlesser & Schultz B-6 nitrocellulose filter and washed 3 times with 2 ml of

saline. Total filtration and wash time was about 10 seconds. Filters were dried and counted by liquid scintillation using toluene-liquifluor (Nuclear-Chicago). Background and non-specific binding were determined by addition of a thousandfold excess of unlabelled TRF to the ^{3}H-labelled material prior to addition to cell membrane fractions.

Results and Discussion:

Binding of ^{3}H-TRF is directly proportional to the cell plasma membrane concentrations over a wide range, limited only by the saturation of the nitrocellulose membrane filters and the amount of time required for filtration and washing to be carried out. Longer wash times on filters removes bound ^{3}H-TRF due to the rapid back reaction (Fig. 3). The ^{3}H-TRF bound is competible by unlabelled TRF (Fig. 1).

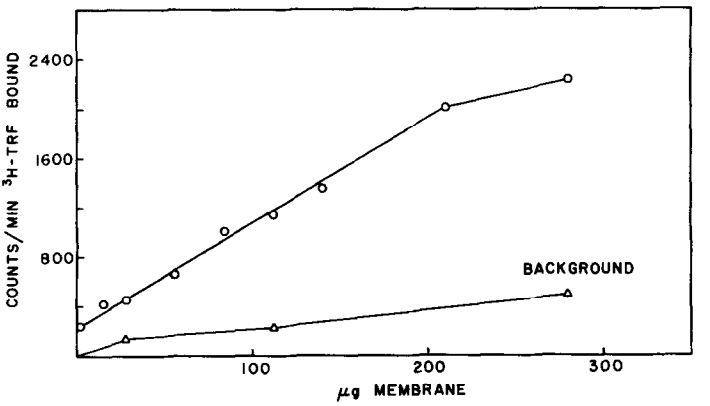

Figure 1. ^{3}H-TRF binding as a function of mouse tumor plasma membrane concentration. Background is established as the non-competible ^{3}H-TRF bound in the presence of a thousandfold excess of unlabelled TRF. ^{3}H-TRF was added to a final concentration of 12 pmoles/ml.

Equilibrium binding of ^{3}H-TRF to plasma membranes, as a function of TRF concentration, is shown (Fig. 2). Saturation of plasma membrane TRF binding receptors is obtained at 1.1 x 10^{-7}M TRF. The apparent dissociation constant is ca. 4 x 10^{-8}M as determined by a Scatchard plot. Calculation of the number of binding sites yields ca. 5 x 10^{8} per micro-

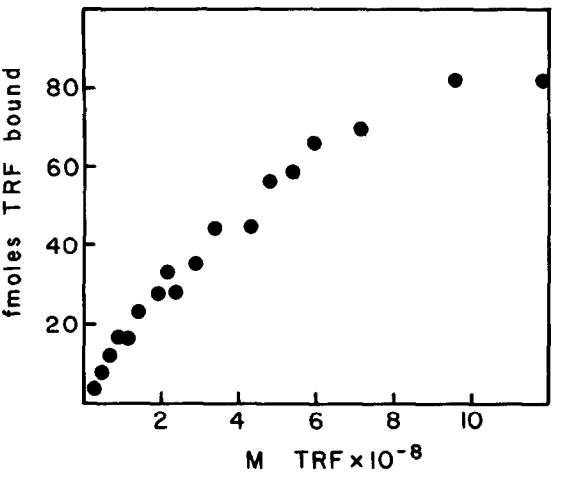

Figure 2. Binding of ^{3}H-TRF as a function of TRF concentration. Non-specific binding (non-competible by unlabelled TRF) has been subtracted (15% of maximal binding). Membrane preparation was used at 150 µg per assay.

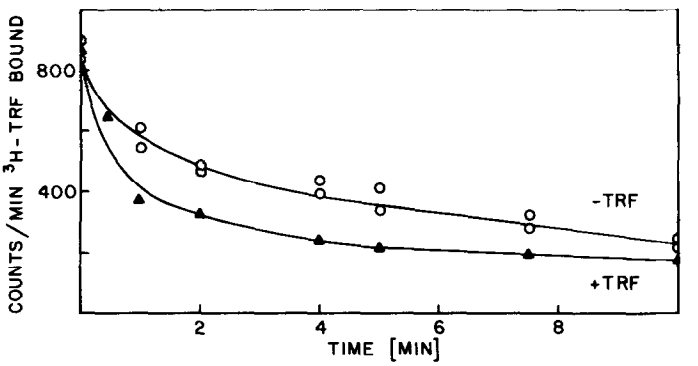

Figure 3. Rate of dissociation of the TRF receptor complex at 0°C in the presence and absence of thousandfold excess of unlabelled TRF. ^{3}H-TRF (12 pmoles/ml) was incubated with 100 µg of membrane for 30 minutes before being either diluted (25-fold) with buffer or competed for by an excess of unlabelled TRF.

gram of plasma membrane. The amount of membrane preparation used limits the maximal amount of ^{3}H-TRF bound to approximately 0.01% of added ^{3}H-TRF, about 85% of which is competible by excess unlabelled TRF.

The dissociation rate of the H-TRF receptor complex at 0°C, by a 25-fold dilution of the reaction mixture into ice cold buffer, gives a 50% dissociation of the complex in about 3 minutes (Fig. 3). The dissociation rate in the presence of excess unlabelled TRF is much more

rapid, 50% dissociation being reached in less than 1 minute. The dissociation does not follow simple first order kinetics and is stimulated by an elevation of TRF concentration. The complexity of this reaction merits further examination.

Stoichiometric competition of binding of ^{3}H-TRF with unlabelled releasing factor is accomplished by choosing a level of ^{3}H-TRF and plasma membranes where all receptors are saturated and ^{3}H-TRF is in slight excess. ^{3}H-TRF (50.4 ng/ml) and increasing concentrations of unlabelled TRF are mixed prior to addition of cell membrane fractions. An exponential dilution curve of c.p.m. bound is obtained (Fig. 4). Receptor affinity is specific for TRF: the inactive TRF analog, pGlu-His-OMe, and synthetic LRF(4) do not compete for the ^{3}H-TRF bound even at 200-fold concentration.

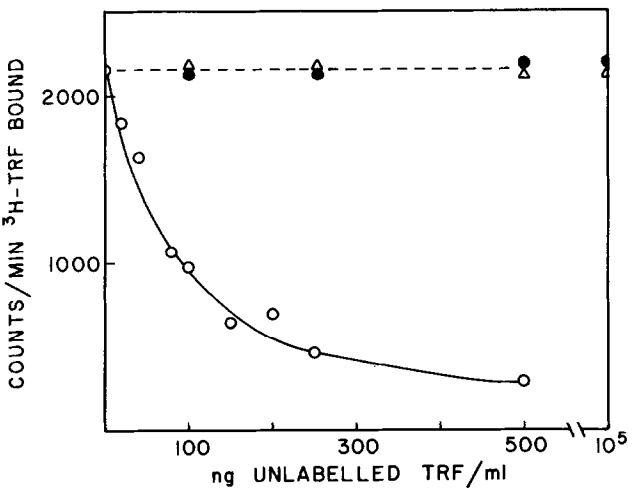

Figure 4. Competition between ^{3}H-TRF and unlabelled TRF for binding to plasma membrane fraction. ^{3}H-TRF (50.4 ng/ml) was mixed with varying concentrations of unlabelled TRF (o——o), added to membrane preparations (1 mg/ml) and incubated for 30 minutes at 0°C before filtration. The ratio of ^{3}H-TRF to unlabelled TRF at 50% competition is 1.3. LRF (▲——▲) and the inactive TRF analog, pGlu-His-OMe, (●——●) do not interfere with binding.

The significance of the binding of ^{3}H-TRF to mouse thyrotropic tumor membranes was tested by determining whether ^{3}H-TRF will bind

to normal or thyroidectomized pituitary cells in tissue culture. Similar equilibrium data were obtained but the amount of binding was much lower than that obtained with tumor preparations, an observation best explained by the low percentage of thyrotrophs present in these preparations. Also, it was shown that ^{3}H-TRF binds to neither plasmacytoma nor neuroblastoma cells grown under identical conditions. The number of TRF binding sites in thyroidectomized pituitary cell populations was ca. 3.75×10^{5} per cell.

Comparison of the binding constant as measured here with that measured using biological activity results in an unexplained discrepancy in that the biological activity yields a constant approximately 20-fold lower than that obtained by the present method (Vale, Grant, Blackwell, Amoss and Guillemin, in preparation). This may be due to the presence of receptors elsewhere in the cell with higher binding affinities, the lack of a requirement to saturate all receptors to yield a maximal biological response or possibly non-physiological conditions of incubation in the present experiments.

The demonstration of TRF receptors on TSH-secreting cells has been presented. Binding and dissociation data indicate a specific reaction with plasma membranes of thyrotrophs. In view of the relatively weak binding constant, the membrane binding technique cannot be used to assay physiological levels of TRF. We are, on the other hand, utilizing this technique to examine the specificity and characteristics of the recognition apparatus in pituitary cells using a series of TRF analogs which have been reported to have varying degrees of biological activity.

ACKNOWLEDGEMENTS: We wish to express our appreciation to Alan Jobe and James Patrick for their advice. Research supported by AID (Contract No. AID/csd 2785), Ford Foundation and Rockefeller Foundation.

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