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Solubilization and Characterization of Pituitary Thyrotropin-Releasing Hormone Receptors

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SUMMARY

Thyrotropin-releasing hormone (TRH) receptors were solubilized from a rat pituitary tumor cell line, GH_4C_1, with digitonin. Convenient assays were developed based on the ability of hydroxylapatite and polyethyleneimine-soaked glass fiber filters to adsorb the solubilized [^{3}H]methyl-TRH-receptor complex but not free [^{3}H]methyl-TRH. The kinetics of [^{3}H]methyl-TRH binding to solubilized receptors were extremely temperature dependent. Binding reached equilibrium at 10–20 nM [^{3}H]methyl-TRH in 30 min at 23° and 6 hr at 0°. The half-times for dissociation were < 5 min at 23° and 7.6 hr at 0°. Equilibrium binding experiments yielded linear Scatchard plots at 0° with K_d = 3 nM, whereas the K_d was > 20 nM at 23°. A series of TRH congeners displaced [^{3}H]methyl-TRH with the rank order reported for membrane receptors, N^3-methyl-HisTRH ≥ TRH > pGlu-N^3-methyl-His-ProNH$(CH_2)_6NH_2$ ≥ pGluHisProNH$(CH_2)_6NH_2$ > pGluHisTyrNH$_2$ ≫ TRH free acid. The antagonist chlordiazepoxide exhibited an IC_{50} of 10 μM. [^{3}H]methyl-TRH binding to solubilized receptors displayed a broad pH optimum, from 6.5 to 7.5. The solubilized receptor could be obtained from cultured GH_4C_1 cells and in much larger quantities from GH_4C_1 tumors. Tumors from 12 rats yielded > 700 pmol of specific soluble TRH binding activity (1 g of protein). The solubilized receptor could be purified 10–20-fold by chromatography on wheat germ agglutinin columns and could be concentrated by adsorption on either DEAE-Sephadex or hydroxylapatite. The procedures outlined allow the solubilization of pituitary TRH receptors from a rich and abundant source, the rapid and reproducible assay of [^{3}H]methyl-TRH binding, and substantial enrichment of receptor activity. These findings should be valuable for the purification and identification of the TRH receptor protein.

TRH binds to high affinity sites in the pituitary gland, brain, and other tissues (1–3). Putative TRH receptors have been characterized in detail in terms of affinity for TRH and related peptides (4–7). Structure-activity data reveal a tight correlation between ability of peptides to displace radioactive TRH or the superagonist methyl-TRH and ability to stimulate TSH and prolactin release. The only apparent antagonist is chlordiazepoxide, which binds competitively to pituitary TRH receptors and shifts the dose-response curve for TRH responses to the right, i.e., chlordiazepoxide behaves as a classical competitive antagonist (8–10). Some differences in affinity of brain and pituitary sites for TRH congeners have been reported (1, 7). To date it is unclear whether these differences are all attributable to differences in metabolism or whether they result from multiple functional classes of TRH receptors.

The properties of TRH receptors have been studied in detail in the anterior pituitary gland, most extensively in prolactin-secreting tumor cell lines (e.g., GH_3, GH_4C_1), which can be grown in culture as clonal populations in high numbers (11–13). Scatchard plots over a broad concentration range are linear, indicating that there is a single affinity class of noninteracting binding sites with a K_d for TRH of 10 mM. Cultured cell lines contain a relatively high density of TRH receptors, 0.5 to 1 pmol/mg of protein, equivalent to around 100,000 sites/cell. TRH binds to surface receptors at all temperatures from 0 to 37° in intact cells. Following the initial binding interaction, a series of temperature-dependent changes ensues rendering the peptide-receptor complex more stable (13).

TRH causes a rapid biphasic increase in the rate of release of stored prolactin and an increase in transcription of the prolactin gene and *de novo* prolactin synthesis (14, 15). The binding of TRH to its receptors stimulates breakdown of phosphatidylinositol-4,5-bisphosphate and leads to an increase in two putative second messengers, inositol triphosphate and diacylglycerol. The inositol triphosphate is believed to lead to release of intracellular calcium, accounting for the observed rapid increase in cytoplasmic free calcium ion, and diacylglycerol is believed to activate protein kinase C, contributing to the second phase of hormone release. Considerable evidence indicates that the TRH receptor interacts with an as yet uniden-

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ABBREVIATIONS: TRH, thyrotropin-releasing hormone; G protein, guanine nucleotide-binding protein; WGA, wheat germ agglutinin; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate.

tified G protein to activate phospholipase C. Guanine nucleotides decrease the affinity of the TRH receptor for agonists by increasing the rate of peptide dissociation (16), and binding of TRH stimulates low K_m GTPase activity in cell membranes (17). These are hallmarks of a receptor interacting with a G protein. None of these features, nor any aspect of the TRH response, is affected by pertussis toxin, indicating that the G protein associated with the TRH receptor is not N_i or N_o (18, 19). Despite considerable progress in describing the molecular events that follow TRH binding, none of the components of the signal transduction system, the TRH receptor, the relevant G protein, or the phospholipase, has been purified yet.

There has been little progress in the purification of TRH receptors from any tissue. Early attempts to solubilize the TRH receptor in an active form were unsuccessful, although the TRH-receptor complex was solubilized with Triton X-100 (12). Johnson *et al.* (20) used digitonin to solubilize active TRH receptors from brain, and the same procedure has been successfully applied to pituitary tissue (21). The solubilized receptor from pituitary cells is guanine nucleotide sensitive if TRH is bound before detergent addition (21). We have been engaged in similar studies characterizing the TRH receptor from the pituitary tumor cell line GH_4C_1, a strain with a high density of TRH receptors. In this paper we report studies that extend previous work on receptors, providing additional data on the properties of digitonin-solubilized receptors, convenient assays, and partial enrichment methods from tumors and cell cultures. These findings should be helpful in the purification procedures necessary for structural characterization of the receptor.

Experimental Procedures

Materials. Tissue culture media and horse serum were purchased from GIBCO (Grand Island, NY) and fetal calf serum from Microbiological Associates (Walkersville, MD). Culture dishes were from Costar (Cambridge, MA) or Corning (Corning, NY). TRH free acid, lectins, detergents, glycine methyl ester, protease inhibitors, nucleotides, ovomucoid, bovine submaxillary mucin, sugars, and buffers were from Sigma Chemical Co. (St. Louis, MO). TRH and pGluHisProNH$(CH_2)_6NH_2$ were gifts from Abbott Laboratories (North Chicago, IL) and chlordiazepoxide from Hoffman LaRoche (Nutley, NJ). pGlu-$N^3{}_x$-methyl-HisProNH$(CH_2)_6NH_2$ was custom-synthesized by Penninsula Laboratories (Belmont, CA). [^{3}H]Methyl-TRH (76.6 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Affi-gel 10, DEAE Sephadex, and hydroxylapatite were from Bio-Rad (Rockville Center, NY).

Growth and maintenance of GH_4C_1 cells. GH_4C_1 cells, a subclone of the GH_3 cell rat pituitary somatomammotrope cell line, were grown in monolayer culture in Ham's F10 medium supplemented with 15% horse serum and 2.5% fetal calf serum, using a humidified atmosphere of 95% air/5% CO_2 as previously described (22).

To obtain large amounts of tumor tissue, the GH_4C_1 cells were injected into female Wistar-Furth rats (100–150 g) using two injections of 0.5–1.0 × 10^7 cells/site. The injections were done in the axillary region, and solid tumors developed in 6–8 weeks. Greater than 90% of the injections resulted in excisable tumors. Rats were sacrificed by CO_2 asphyxiation and the tumors were surgically removed and washed in saline. Excess fat and connective tissue were removed and the tumors were then used for the solubilization of the TRH receptor. The tissue was minced and homogenized in a glass vessel with power-driven pestle in TM buffer (20 mM Tris·HCl, 2 mM $MgCl_2$, pH 7.5), with 1 mM EDTA and 5 μg/ml soybean trypsin inhibitor, using approximately 3 ml/g of tumor tissue and was filtered through two layers of cheesecloth. The suspension was treated at 750 psi for 10 min in a Parr disruption bomb and processed as described in the text.

GH_4C_1 cells were counted with a hemocytometer. Protein concentration was determined by the method of Lowry *et al.* (23). For those solutions that contained detergents, the protein was measured by a modification of the Lowry procedure, which employed a trichloroacetic acid precipitation step before the reaction (24).

Solubilization and assay of TRH receptors. For routine solubilization of TRH receptors, GH_4C_1 membranes were washed with 0.1% digitonin in TM buffer and then suspended in a 1% digitonin (w/v) solution in TM buffer. The solution was stirred at 4° for 30 min and centrifuged at 100,000 × *g* for 45 min. The supernate was collected; this contained solubilized TRH receptors. Individual lots of digitonin differed in their ability to solubilize active TRH receptors and batches were screened before use.

The superagonist [^{3}H](*N*-3-methyl-His)TRH (K_d = 2 nM) was used to assay TRH receptors. Binding to intact cells and membranes was measured as previously described (11). Nonspecific binding was determined in parallel reactions containing 1 μM unlabeled TRH. This was less than 5% of the total counts and has been subtracted from all data unless otherwise indicated.

Several methods were used to assay high affinity binding to the soluble TRH receptor. Filtration through Sephadex G75 was used to separate [^{3}H]methyl-TRH associated with the high molecular weight receptor from the free ligand. Parallel columns were run for reactions that included excess unlabeled TRH. The difference in the amount of radioactivity contained in high molecular weight peaks represented the specific binding of [^{3}H]methyl-TRH to the soluble receptor. Nonspecific binding was < 5% of total.

In order to assay multiple points, rapid gel filtration under centrifugal force was used (25). Plastic 5-ml syringes were fitted with 3-mm glass beads and filled with 3 ml of Sephadex G75 swollen in TM buffer with 0.1% digitonin. The tubes were drained, placed in 12-ml plastic centrifuge tubes, and spun in a clinical IEC centrifuge for 3 min on Setting 2. The binding reaction was brought to 0.5 ml with 0.1% digitonin in TM buffer at 0°, layered over the prespun gel, and centrifuged again. The eluate contained receptor-bound [^{3}H]methyl-TRH. This procedure gave the same amount of binding as standard gel filtration but tended to result in more variability and higher blanks.

A second assay involved batch hydroxylapatite adsorption. At 10% (w/v) hydroxylapatite suspension was made in TM buffer with 0.1% digitonin and 10 mM $CaCl_2$ (TMDC buffer). Hydroxylapatite suspension (0.5 ml) was added to each tube at 0°. After 10 s to 2 min, 2 ml of ice-cold TMDC buffer was added, and the tubes were vortexed and centrifuged at 1000 × *g* for 1 min. In some experiments a second identical wash was added. The pellet was resuspended in 2 ml of TMDC buffer and the centrifugation was repeated. One milliliter of 99% ethanol was added to elute receptor-bound [^{3}H]methyl-TRH, and 1 to 15 min later the tubes were centrifuged and an aliquot of the supernate was counted.

A third assay was filtration through polyethyleneimine-soaked filters (26). GF/A filters were soaked at 4° in 0.3% polyethyleneimine in water for 1 hr to 2 months. Reaction mixtures were diluted to 3 ml with TM buffer containing 0.1% digitonin and filtered through the polyethyleneimine-soaked filters. Filters were washed two or three times in the same buffer, dried, and counted.

Unless noted, [^{3}H]methyl-TRH binding to soluble receptors was measured as follows. Reactions were performed in a final volume of 100 or 200 μl containing 100–200 μg of protein from the S3 fraction, as described in Tables 2 and 3, and 10 nM [^{3}H]methyl-TRH. This concentration of [^{3}H]methyl-TRH is 3 times the K_d and should saturate 75% of receptors. Nonspecific binding was determined using identical reaction mixtures that contained a 100-fold excess of nonradioactive TRH. Tubes were incubated for 18 hr at 0–4° and the hydroxylapatite procedure was used to separate receptor bound from free [^{3}H]methyl-TRH. Data are corrected for nonspecific binding. Unless otherwise noted, membrane binding was measured as described previously (11) using 10 nM [^{3}H]methyl-TRH and a 1-hr incubation at 20°.

Synthesis of Affinity Matrices. WGA was covalently coupled to agarose using the activated support Affi-gel 10, which contains side chains off the polymer backbone that terminate with *N*-hydroxysucci-

nimide groups, as previously described (27). WGA was dissolved in buffer (0.2 M sodium carbonate, pH 8.4, 2 mM $MnCl_2$, 2.0 mM $CaCl_2$, and 10% (w/v) *N*-acetyl-D-glucosamine) at a concentration of 5–10 mg/ml. The WGA solution was mixed with an equal volume of Affi-gel 10 and equilibrated with the same buffer for 2 hr at room temperature, then overnight at 4°. The remaining reactive groups were blocked by adding 0.1 M glycine methyl ester in the coupling buffer and mixing for 1 hr at room temperature. The WGA-Sepharose was washed and stored at 4° in the coupling buffer supplemented with either 0.02% thimerosal or 0.2% sodium azide.

Results

Assays for solubilized TRH receptors. Three assays were used to measure binding of [^{3}H]methyl-TRH to digitonin-solubilized receptors (Table 1). Separation by centrifugation through Sephadex G75 columns provided consistent results and this was the method we used in initial experiments to define solubilization procedures. However, the column method was difficult to apply to large numbers of samples and blanks were substantial at higher [^{3}H]methyl-TRH concentrations. We developed two other convenient assays applicable to multiple tubes. A batch procedure was developed taking advantage of the fact that the solubilized TRH receptor, but not free [^{3}H]methyl-TRH, adsorbs to hydroxylapatite. A slurry of hydroxylapatite (10% v/v) was added to reaction mixtures. Each 1 ml of packed volume quantitatively bound TRH-binding activity in 2.8 mg of solubilized GH_4C_1 cell protein. Additional buffer was added, the tubes were centrifuged briefly, and the hydroxylapatite pellet was washed; receptor-bound [^{3}H]methyl-TRH was eluted with ethanol. Another method, based on a published procedure for detergent-solubilized receptors (26), was to filter reaction mixtures through glass fiber filters soaked in polyethyleneimine. Receptor-bound but not free [^{3}H]methyl-TRH was retained on the filters. Both the hydroxylapatite and filtration methods gave values close to those obtained by gel filtration and typical errors of less than ±5%. Nonspecific binding was about 0.5–1% of added counts and was virtually identical to blanks obtained with [^{3}H]methyl-TRH in the absence of protein. This indicates that the nonspecific association of [^{3}H]methyl-TRH with solubilized GH_4C_1 cell proteins, as with GH_4C_1 cell membranes, is negligible (11).

Solubilization procedures. Digitonin solubilized TRH receptors in active form from GH_4C_1 cells in culture and from GH_4C_1 tumor tissue. The yield of soluble TRH receptor was maximal at 1% (w/v) digitonin, which represented 4 mg of digitonin/mg of protein. Typical yields of solubilized receptors from GH_4C_1 cell cultures are shown in Table 2. Cells were routinely lysed by homogenization in hypotonic buffer, centrifuged at low speed to remove debris, and washed with a low concentration of digitonin. TRH receptors were then solubilized with 1% digitonin. Receptors were solubilized in approximately 50% yield with 3-fold enrichment over crude homogenates.

Tumors derived from GH_4C_1 cells provided a source of much larger quantities of TRH receptors than those easily obtained from cell culture. As much as 14 g of tumor could be obtained from each rat. Table 3 shows the results of a representative preparation of soluble TRH receptors from one set of tumor-bearing rats. The homogenate contained 884 pmol of TRH receptor, with a very low apparent specific binding activity (27 fmol/mg of protein). The solubilization procedure was quite successful in removing much of the non-receptor protein without sacrificing yield and the final digitonin-solubilized fraction was 28-fold enriched in TRH binding activity, with a calculated yield of 88%. At this stage, the specific TRH binding activity was half that obtained from cultured cells.

The soluble receptor prepared from cultured cells or tumors was stable when frozen at −20° or −70°, with only slight loss of activity up to 6 months. This stability was identical for receptors solubilized from GH_4C_1 cells grown in culture or tumors. The soluble receptor retained at least 90% of its activity at 0–4° for up to 1 week and was stable during several days of dialysis. Although we routinely included protease inhibitors, we did not find evidence of rapid proteolysis in the digitonin-solubilized preparations. This is in contrast to the more rapid degradation of TRH receptors in membrane preparations.

Characterization of the soluble TRH receptor. Specific binding of 15 nM [^{3}H]methyl-TRH to the soluble receptor reached equilibrium in 6 hr at 0° and in only 30 min at 23° (Fig. 1). Once equilibrium was attained, there was no decline in the amount of specific [^{3}H]methyl-TRH binding over 18 hr at 0° (data not shown). This result indicates that the peptide receptor complex, like the free receptor, is quite stable. [^{3}H]Methyl-TRH was in great excess over receptor in these experiments. First-order plots of the approach to equilibrium data (Fig. 1, *insets*) give values for $k_{obs} = 0.08$ min^{-1} at 0° (15 nM [^{3}H]methyl-TRH) and $k_{obs} = 0.16$ min^{-1} at 23° (20 nM [^{3}H]methyl-TRH). The half-times for dissociation of [^{3}H]methyl-TRH from soluble receptors was 7.6 hr at 0° and less than 5 min at 23° (Fig. 1). First-order plots of the dissociation data (Fig. 1, *insets*) were not strictly linear.

Equilibrium binding of [^{3}H]methyl-TRH to solubilized receptors was measured at 0° and 23°. Scatchard plots of data obtained at 0° were linear and gave an apparent K_d of 3 nM (Fig. 2). K_d values, at 0°, between 3 and 6 nM have been obtained for receptors solubilized from cells and tumors before and after partial purification (see below). At 23°, [^{3}H]methyl-TRH binding did not saturate at concentrations up to 20 nM (Fig. 2). This difference in binding was not due to irreversible inactivation of the receptor inasmuch as binding increased 3-fold if

TABLE 1

Assay of solubilized receptors

Digitonin-solubilized TRH receptors from cell cultures (S3 fraction) in 200 μl were incubated with 10 nM [^{3}H]methyl-TRH (50,000 dpm) and assayed by either the rapid filtration, polyethyleneimine, or hydroxylapatite procedures described under Experimental Procedures. Hydroxylapatite was washed twice. Nonspecific binding refers to binding in the presence of 1 μM unlabeled TRH, whereas the blank represents values obtained with [^{3}H]methyl-TRH but no receptor.

Procedure	Total binding	Nonspecific binding	Blank	Specific binding
		dpm		
Sephadex	12,000 ± 523	1,020 ± 52	ND*	9,330 ± 525
Hydroxylapatite	8,680 ± 231	177 ± 16	106 ± 16	8,502 ± 232
Polyethyleneimine filters	8,302 ± 215	479 ± 21	467 ± 16	7,823 ± 216

* ND, not determined.

TABLE 2

Preparation of solubilized TRH receptors from cultured cells

Monolayer cultures of GH_4C_1 cells (total growth surface, 1500 cm^2) were harvested, centrifuged, and homogenized in TM buffer with 25 strokes in a Dounce homogenizer. Broken cells were centrifuged (1000 × *g* for 1 min) and the supernatant (S1) brought to 0.1% digitonin, incubated 10 min at 0° and centrifuged at 100,000 × *g* for 1 hr. The pellet (P2) was homogenized five strokes with 1% digitonin, incubated at 0° for 30 min, and centrifuged at 100,000 × *g* for 45 min. The supernatant (S3) contained solubilized TRH receptors. Specific binding of 10 nM [^{3}H]methyl-TRH was measured in 1-hr (membrane fractions) or 18-hr (soluble fractions) incubations as described under Experimental Procedures.

Fraction	Protein	Volume	Specific binding	Total binding	Yield
	mg	*ml*	*pmol/mg*	*pmol*	%
Cell suspension	39.8	10.9	0.54	21.4	100
S1 (1000 × *g* supernate)	32.9	10	0.80	26.3	123
P1 (1000 × *g* pellet)	1.09	1	0.50	0.54	3
S2 (100,000 × *g* supernate) (0.1% digitonin)	8.54	11	0.0	0.0	0
P2 (100,000 × *g* pellet)	24[a]	0.5	0.42	10.4	48
S3 (100,000 × *g* supernate) (1% digitonin)	6.51	10.5	1.50	9.85	46

[a] Calculated from S1 − S2 protein values.

TABLE 3

Preparation of solubilized TRH receptors from tumors

Tumors (137 g wet weight) from 12 Wistar Furth rats were obtained and processed as described under Experimental Procedures. The homogenate was centrifuged at 400 × *g* for 1 min and the pellet was washed once. The combined supernates (S1) were brought to 0.2% digitonin/1 mM EDTA, incubated 10 min, and centrifuged at 100,000 × *g* for 20 min. TRH receptors were solubilized from the pellet (P2) with 1% digitonin as described for cells. Specific binding of 10 nM [^{3}H]methyl-TRH was measured in 1-hr (membrane fractions) or 18-hr (soluble fractions) incubations as described under Experimental Procedures.

Fraction	Protein	Volume	Specific binding	Total binding	Yield
	mg	*ml*	*fmol/mg*	*pmol*	%
Homogenate	32,200	375	27.0	884	100
S1 (400 × *g* supernate)	27,100	360	14.0	377	43
P1 (400 × *g* pellet)	4,900	45	2.0	10.2	1.2
S2 (100,000 × *g* supernate) (0.2% digitonin)	21,300	440	0.5	5.5	0.1
S3 (100,000 × *g* supernate) (1% digitonin)	1,090	470	719	783	88

tubes were chilled to 0° after incubation at 23°. This behavior is the same as that observed for TRH binding to membranes, although binding in intact cells is not temperature dependent (28).

The optimum pH for the binding reaction was 6.5–7.5 (Fig. 3). TRH receptors were not irreversibly inactivated at pH 5.5, because over 60% of binding activity could be recovered if the pH was raised to 7.5 by addition of buffer and over 90% recovered if neutralization was followed by dialysis to remove salt. The pH profile for the digitonin-solubilized receptor is the same as that for the membrane receptor (11).

The pharmacological specificity for solubilized TRH receptors was tested in competition displacement experiments measuring the potency of various peptides to displace [^{3}H]methyl-TRH (Fig. 4). TRH and methyl-TRH were the most potent analogs (IC_{50} = 3 and 30 nM), whereas the C-terminally extended peptides pGlu-N^3-methyl-HisProNH$(CH_2)_6NH_2$ and pGluHisProNH$(CH_2)_6NH_2$ exhibited IC_{50} values of about 400 and 100 nM, respectively. The analog *p*GluHisTyrNH_2 had a much higher IC_{50} (10 μM) and the free acid of TRH was only slightly inhibitory at concentrations as high as 0.1 mM. The TRH antagonist chlordiazepoxide displaced TRH with an IC_{50} of 10 μM. The competition displacement curves were shallower than expected for TRH and methyl-TRH and the reasons are unclear.

Binding in TM buffer (15 hr at 4°) was inhibited by salts and nucleotides with IC_{50} values as follows: 400 mM KCl, 180 mM NaCl, 100 mM $MgCl_2$, and >1 mM GTP and Gpp(NH)p. These findings agree with the report that the TRH receptor isolated without bound ligand is insensitive to guanine nucleotides (21).

We were unable to solubilize active receptors with other detergents, including cholate, Lubrol WX, or CHAPS as well as those previously reported (12). Furthermore, when digitonin-solubilized receptors were dialyzed and other detergents added, binding activity was lost (Fig. 5). We were unable to reverse the inactivation by cholate or CHAPS by dialyzing solubilized receptors into digitonin. These results precluded exchanging digitonin for a detergent with more favorable hydrodynamic properties for characterization of the free receptor.

Chromatography of solubilized TRH receptors. The ability of various lectins to absorb solubilized TRH receptors was tested using 15 mg of lectin attached to Sepharose (0.5–1.1 ml) per mg of solubilized receptor. WGA-Sepharose adsorbed 91% of the applied soluble receptor whereas concanavalin A and lentil lectin were much less effective, absorbing 42% and 28% of binding activity, respectively. The ability of various compounds to inhibit the adsorption of solubilized TRH binding activity to WGA-Sepharose was tested. IC_{50} values were (w/v): 4% ovomucoid, 3% *N*-acetyl-D-glucosamine, > 10% bovine submaxillary mucin and *N*-acetyl-D-galactosamine.

Lectin chromatography was used to enrich TRH-binding activity. WGA-Sepharose columns rapidly adsorbed 80–90% of the applied soluble receptor. Receptor was eluted from the column in reasonably high yield (62%) with 0.5 M *N*-acetyl-D-glucosamine, with an overall enrichment of binding activity in different experiments of 10- to 20-fold (Fig. 6). Sugar could be removed by dialysis or by concentrating receptor on hydroxylapatite, although this was not found to be necessary for routine assay. The equilibrium [^{3}H]methyl-TRH-binding characteristics of the WGA-Sepharose eluate were identical to those

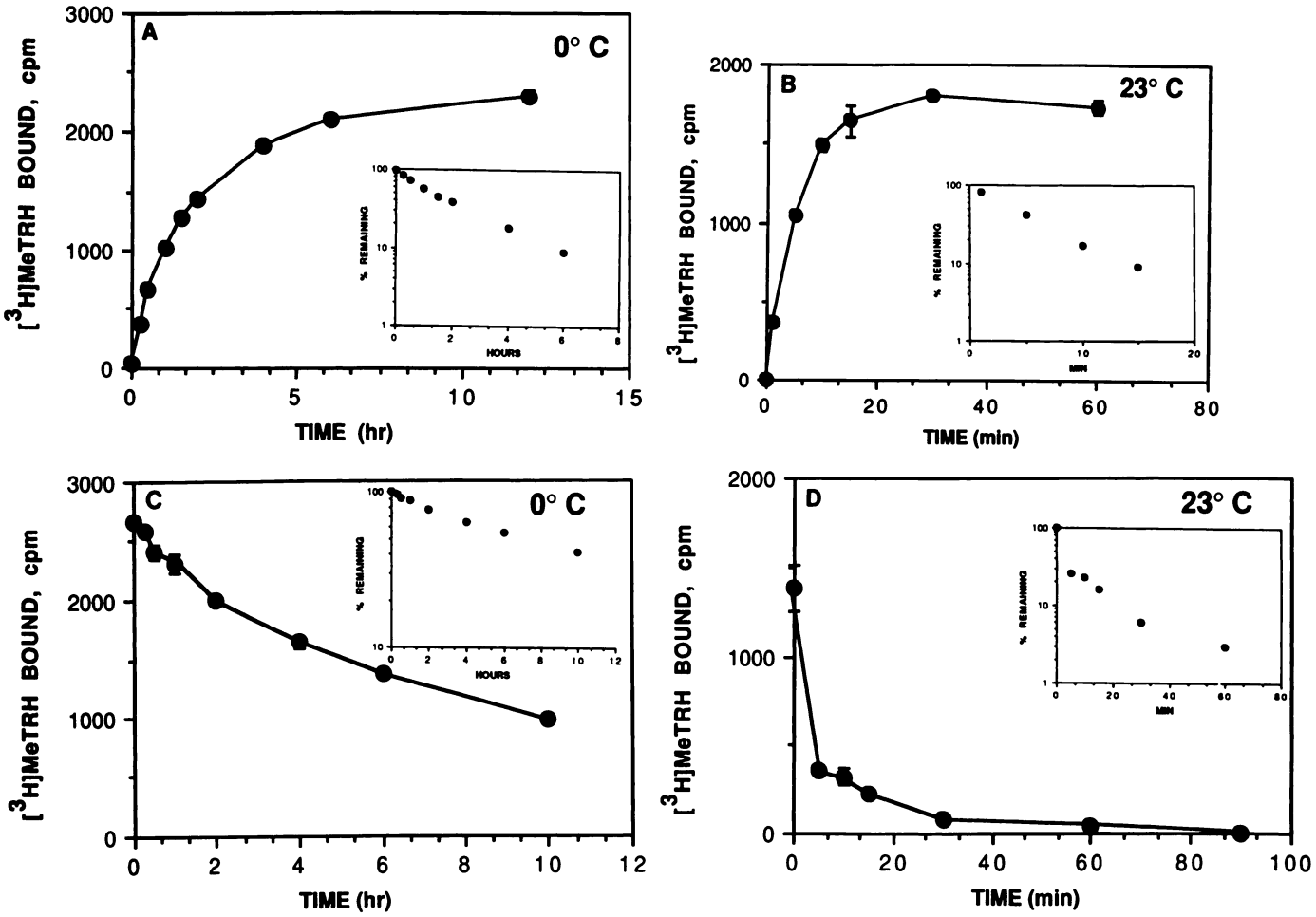

Fig. 1. Kinetics of [^{3}H]methyl-TRH to solubilized receptors. A and B, Association rates. Digitonin-solubilized receptors (S3 fraction from a tumor preparation, 3 mg/ml) were incubated with 15 nM (0°) or 20 nM (23°) [^{3}H]methyl-TRH and the amount of specifically bound [^{3}H]methyl-TRH was measured at intervals. C and D, Dissociation rates. Receptors were incubated with 10 nM [^{3}H]methyl-TRH for 24 hr at 0° or with 20 nM [^{3}H]methyl-TRH for 1 hr at 23°, then 10 μM unlabeled TRH was added, and specifically bound radioligand was measured at intervals using the hydroxylapatite procedure. The *insets* show first-order plots of the percentage of sites available (association curves) or occupied (dissociation curve). The S3 fraction from a tumor preparation containing 3.4 mg of protein/ml was used for these experiments. Values are the means of triplicate determinations, which agreed within 10%.

obtained for the crude solubilized receptor with a linear Scatchard plot and a K_d of 3.5 nM.

DEAE-Sephadex bound a significant amount of soluble protein including the TRH receptor (Fig. 7). The peak of the receptor eluted at 75 mM KCl, as did the peak of protein, and recovery of TRH binding activity was high, >90%. Soluble receptor as well as a majority of the other soluble proteins bound to hydroxylapatite and active receptor could be eluted in a broad peak with high concentrations of phosphate (200 mM or greater) (data not shown). Both DEAE and hydroxylapatite columns could be used as concentration steps, although they provided little enrichment. SP-Sephadex did not bind the soluble receptor. TRH binding activity eluted from Sepharose 6B in an asymmetric peak with an apparent Stokes radius of 50Å (Fig. 8).

Discussion

A growing number of cell surface receptors have been cloned and can be classified on the basis of their structure as well as their function. Receptors with tyrosine kinase activity such as those for insulin, platelet-derived growth factor, and epidermal growth factor are characterized by a single membrane-spanning domain and can typically be solubilized by detergents like Triton X-100 (29). These receptors do not appear to interact directly with G proteins, although some controversy surrounds this point. Another class of receptors includes the α- and β-adrenergic receptors, muscarinic receptors, and rhodopsin (30). These receptors consist of single polypeptide chains that span the plasma membrane seven times and usually require digitonin or other detergents with sterol ring structure for solubilization. All of these receptors have been shown to interact with G proteins. Based on its interaction with a G protein and the requirement for digitonin for solubilization, it seems likely that the TRH receptor falls into this latter class. The TRH receptor probably contains *N*-linked carbohydrate residues, because it is adsorbed by WGA and lectin chromatography provided a 10--20-fold enrichment of binding activity. Multiple receptor subtypes are a common finding, and brain and pituitary TRH receptors may exhibit differences in preference for some analogs (1, 7). In this regard, it is of interest that the properties of the solubilized pituitary receptor including apparent Stokes radius are very close to those reported for the TRH receptor solubilized from brain (20).

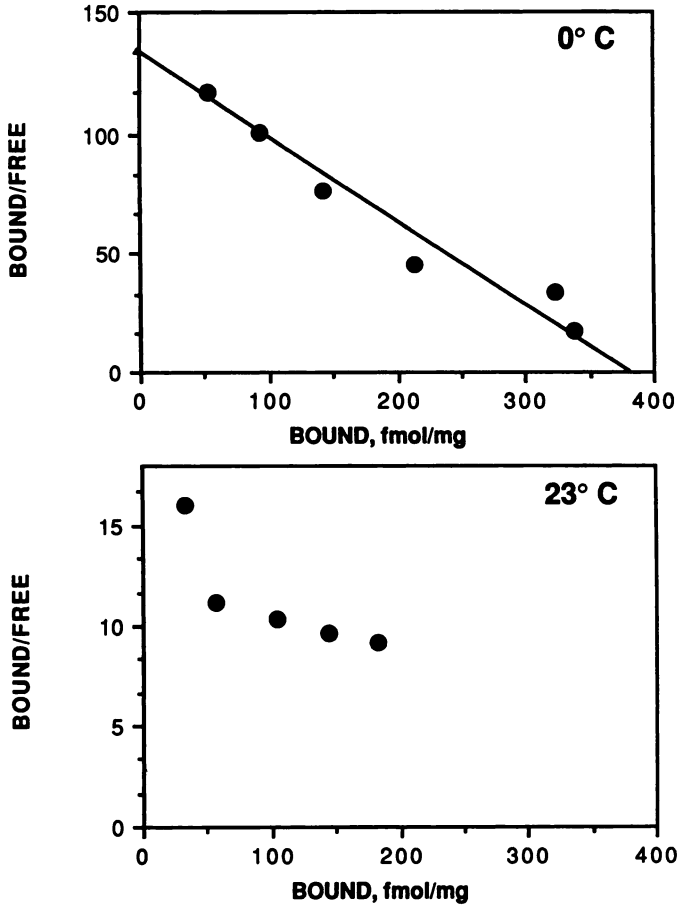

Fig. 2. Equilibrium binding of [^{3}H]methyl-TRH. Solubilized receptors were incubated with different concentrations of [^{3}H]methyl-TRH for 18 hr at 0° or 1 hr at 23° and specific binding was measured by the rapid gel filtration method; 180 μg of protein from the S3 fraction were used in each 200-μl reaction. At 0°, K_d = 3.1 nM and B_{max} = 390 fmol/mg of protein. When binding of 20 nM [^{3}H]methyl-TRH binding was measured at different temperatures in the same experiment, there was 32% as much specific binding at 23° as at 0°.

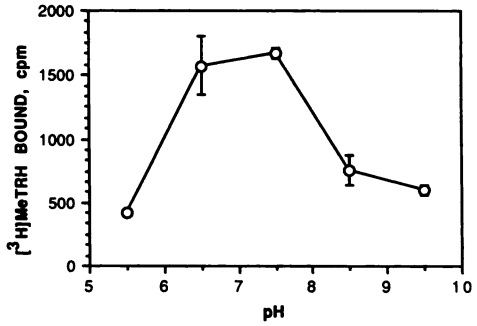

Fig. 3. pH dependence of [^{3}H]methyl-TRH binding to solubilized receptors. Receptors solubilized in 5 mM potassium phosphate buffer at pH 7 were brought to the indicated pH with 35 mM potassium phosphate and incubated with 5 nM [^{3}H]methyl-TRH for 18 hr before specific binding was measured by the rapid gel filtration method.

Because the TRH receptor is present in GH_4C_1 cells at a concentration of about 0.5 pmol/mg of cell protein then, if an estimate of 100,000 is used for the molecular weight of a binding site, a 20,000-fold purification will be required to yield pure receptor and the digitonin-solubilized receptor is 0.1–1% pure after the lectin chromatography step. We have bound receptor at this stage to affinity columns containing pGluHis-

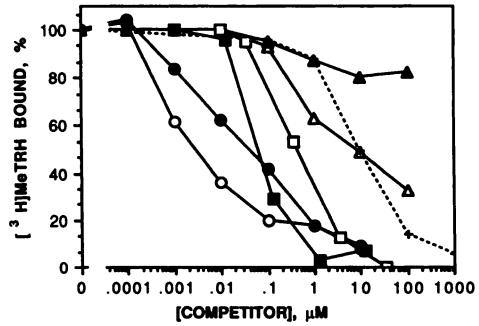

Fig. 4. Pharmacological specificity of solubilized TRH receptor. Digitonin-solubilized receptor was incubated for 18 hr at 0° with 5 nM [^{3}H]methyl-TRH and peptides as shown. The data for TRH and [N^3-methyl-His]^{2}TRH are the mean of seven determinations in three separate experiments; other *points* show the mean of specific binding of duplicate determinations in a single experiment, which agreed within ± 10%. Nonspecific binding has not been subtracted. ●, TRH; ○, [N^3-methyl-His]^{2}TRH; ■, pGluHisProNH$(CH_2)_6NH_2$; □, pGlu-N^3-methyl-HisProNH$(CH_2)_6NH_2$; △, pGluHisTyrNH$_2$; ▲, TRH free acid; and +, chlordiazepoxide.

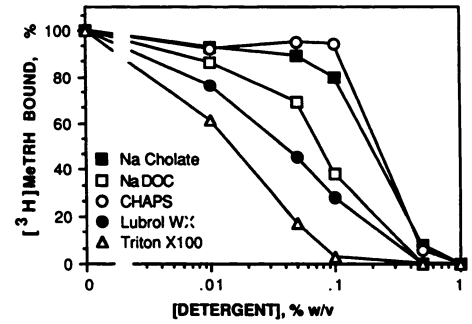

Fig. 5. Effect of detergents on soluble receptor activity. Digitonin-solubilized receptors were dialyzed overnight in TM buffer without detergent. Aliquots were incubated with detergents as shown and the specific binding of 6 nM [^{3}H]methyl-TRH was measured after an 18-hr incubation at 4°.

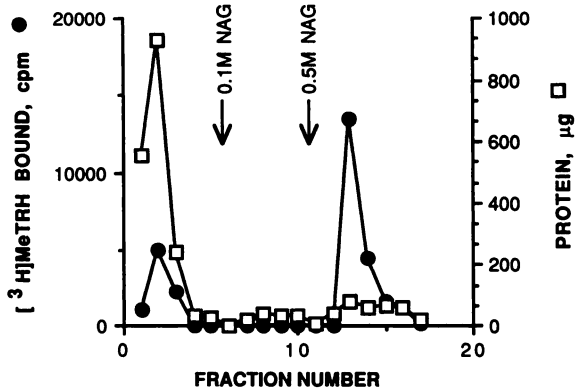

Fig. 6. WGA chromatography of soluble TRH receptors Digitonin-solubilized TRH receptor was loaded onto a 5-ml WGA-Sepharose column for 2 hr at 4° and the column was washed with 20 ml of TM buffer containing 0.1% digitonin. At the time shown by the *first arrow*, 0.1 M *N*-acetyl-D-glucosamine was added in the same buffer and, at the time shown by the *second arrow*, 0.5 M *N*-acetyl-D-glucosamine was added. Fractions were analyzed for specific [^{3}H]methyl-TRH binding and protein.

ProNH$(CH_2)_6NH_2$ bound to Affi-gel 10 but were not able to elute more than 10% of the binding activity with TRH, chlordiazepoxide, low pH, or high salt. We cannot determine whether this failure resulted from tight binding, irreversible denaturation, or possibly the loss of a necessary protein or small molecule. When we elute material bound to the affinity column

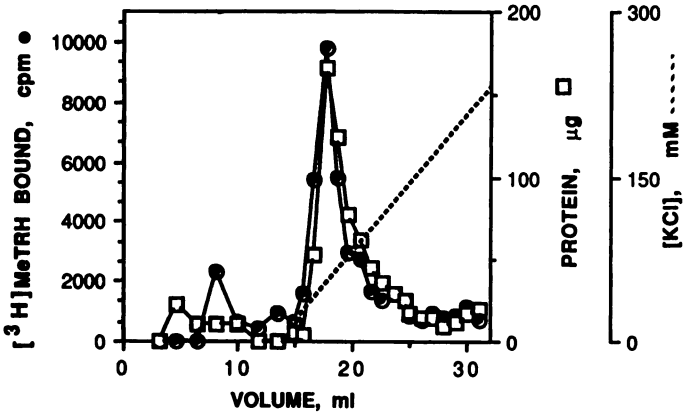

Fig. 7. DEAE-Sephadex chromatography of soluble TRH receptors. Digitonin-solubilized TRH receptor was loaded onto a 3-ml DEAE-Sephadex column equilibrated in 20 mM Tris·HCl, 0.4% digitonin pH 7.5. The column was washed in this buffer and then a 0–0.4 M KCl gradient was applied in the same buffer and specific binding of 10 nM [^{3}H]methyl-TRH was measured.

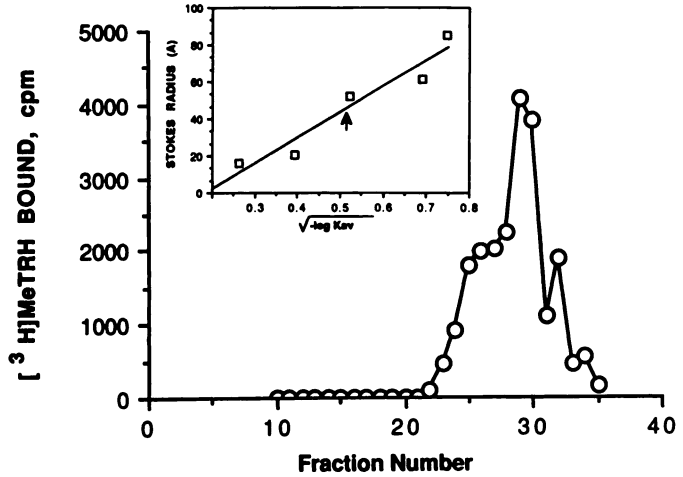

Fig. 8. Gel filtration of the soluble TRH receptor. Soluble receptor (0.5 ml) was loaded onto a 24-ml Sepharose 4B column equilibrated in TM buffer with 0.1% digitonin at 4° and the column was run at 0.12 ml/min. Specific binding of 10 nM [^{3}H]methyl-TRH was measured for each fraction. The *inset* shows calibration of the column using methyl-cytochrome *c* (16.4 Å), myoglobin (20.7 Å), catalase (52 Å), ferritin (61 Å), and thyroglobulin (85 Å). K_e was determined as described (31). The *arrow* shows the elution of the peak of TRH receptor activity.

under denaturing conditions and radioiodinate, the protein we obtain has a predominant band at about 70,000 Da, which is not contaminating albumin, but we are uncertain whether this band is the TRH receptor.

Biochemical purification is critically dependent on the availability of a good receptor source and convenient and reliable assays. We have developed two simple inexpensive procedures that are applicable to multiple samples, give low nonspecific binding, and are highly reproducible. We have also developed procedures to solubilize receptors from pituitary tumors, which provide gram rather than milligram quantities of receptor-rich tissue and should reduce the cost of receptor purification by several orders of magnitude. The yield of receptor from tumors from 12 rats provided as much material as over 500 100-mm culture dishes. This work complements previous studies on the TRH receptors and should contribute to the eventual identification of the protein.

Purification of the receptor is an important step in understanding the molecular mechanism of TRH action. Biochemical quantities of receptor are needed for physical characterization and purified receptor should allow antibody formation, which is a prerequisite to localizing TRH receptors. This should prove of particular value in tissues such as brain, spinal cord, pancreas, and retina in which the physiological function of the peptide is unclear. The methods presented in this paper should also prove useful for the eventual cloning of the receptor. The properties of the solubilized receptor from pituitary cells and tumors in terms of pH and salt dependence, molecular size, and pharmacological specificity are similar to those reported for the membrane receptor (2, 11) and to those reported for brain and pituitary solubilized receptors (20, 21). Because the solubilized receptor retains normal ability to interact with ligands and with G proteins, it should be useful in reconstitution studies directed towards understanding the signal transduction pathway.

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