Synthesis and characterization of a high-affinity photoactivatable analogue of thyrotropin-releasing hormone

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An analogue of thyrotropin-releasing hormone (TRH, pGlu-His-ProNH₂), i.e. pGlu-His-ProNH-(CH₂)₆-(4-azido-salicylamide) (TRH-ASA), has been synthesized and, in a radioiodinated form (TRH-IASA), characterized and used as a photoaffinity reagent to label the TRH receptor on rat pituitary GH₄C₁ cells. TRH-IASA bound to GH₄C₁ cells with high affinity ($K_d = 8$ nM), comparable with that of TRH binding. The binding of TRH-IASA was competitive with binding of TRH, two TRH analogues and a TRH receptor antagonist, chlordiazepoxide. TRH-IASA did not bind to or label GH₁2C₁ cells, which lack functional TRH receptors. Labelling of GH₄C₁ cells with TRH-IASA followed by SDS/PAGE and autoradiography of membrane proteins demonstrated labelling of a single polypeptide which ran as a diffuse band between 71 and 91 kDa, centred at 76 kDa. No change in this labelling pattern was observed as a function of the length of time (between 5 min and 2 h) that GH₄C₁ cells were incubated with 3 nM-TRH-IASA. Using either a very short (5 s) photolysis interval or low TRH-IASA concentrations, only the 76 kDa band was labelled. Minor bands appeared only after extended photolysis and use of high TRH-IASA concentrations. We conclude that the TRH receptor from rat pituitary GH₄C₁ cells is a single peptide with an apparent molecular mass of 76 kDa. Details of the chemical synthesis of TRH-ASA are given in Supplementary Publication SUP 50167 (5 pages), which has been deposited at the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in Biochem. J. (1992) 281, 5.

INTRODUCTION

Thyrotropin-releasing hormone (TRH, pGlu-His-ProNH₂) is a hypothalamic tripeptide which stimulates thyrotropin and prolactin secretion and biosynthesis in the pituitary gland (MacLeod *et al.*, 1988), and performs neuromodulatory functions in the central nervous system (Johnson *et al.*, 1989). The clonal rat pituitary GH₄C₁ cell strain (Tashjian *et al.*, 1968; Tashjian, 1979) expresses plasma membrane receptors for TRH (Hinkle & Tashjian, 1973; Hinkle *et al.*, 1974) and responds to TRH with rapid and sustained secretion of prolactin (Dannies & Tashjian, 1976; Albert & Tashjian, 1984a; Hinkle, 1989) and with *de novo* prolactin synthesis (Dannies & Tashjian, 1976; Murdoch *et al.*, 1983).

GH cell strains have been popular models for the study of receptor-mediated signal transduction (Drummond, 1986; Hinkle, 1989; Tashjian, 1990). In GH cells, TRH receptor occupancy stimulates the formation of inositol 1,4,5-trisphosphate and diacylglycerol (Martin, 1983; Imai & Gershengorn, 1985) and a biphasic increase in cytosolic free Ca²⁺ (Albert & Tashjian, 1984b). The TRH receptor exhibits unusual ligand-binding kinetics (Hinkle & Kinsella, 1982), no apparent desensitization (Hinkle, 1989), and affinity modulation by specific lipids (Winicov *et al.*, 1990), Na⁺ and guanyl nucleotides (Hinkle & Kinsella, 1984).

Reported synthetic TRH analogues (Hinkle *et al.*, 1974) include a fluorescein-tagged molecule (Halpern & Hinkle, 1981), numerous analogues which partially discriminate between the endocrine and central nervous system functions of TRH (e.g. Szirtes *et al.*, 1986), potential affinity analogues based on diazomethane or chloromethane moieties substituting for the amide terminus (Balk *et al.*, 1981), and a photosensitive TRH

analogue containing 2-diazohistidine (Meyer *et al.*, 1991). Proteins previously reported as possible candidates for the TRH receptor include a 70 kDa protein tentatively identified by affinity chromatography (Phillips & Hinkle, 1989), and a 64 kDa peptide weakly labelled after irradiation of GH cell membranes pre-incubated with [³H]TRH (Wright *et al.*, 1988). We report here the synthesis, characterization and first application of a high-affinity, radiolabelled, photoactivatable analogue of TRH.

EXPERIMENTAL

Materials

Tissue culture plasticware was from Falcon Labware (Division of Becton, Dickinson and Co., Lincoln Park, NJ, U.S.A.). Culture medium and sera were from Gibco Laboratories (Grand Island, NY, U.S.A.). TRH was purchased from Peninsula Laboratories, Inc. (Belmont, CA, U.S.A.). [³H]*N*⁷-methyl-TRH and ¹²⁵I were obtained from NEN Research Products (Boston, MA, U.S.A.). Solvents (ACS Grade) were purchased from either Fisher Scientific (Pittsburgh, PA, U.S.A.) or Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). 1,6-Diaminohexane, di-*t*-butyl dicarbonate, palladium on carbon, diatomaceous earth, anhydrous methanol, tetrahydrofuran and methylene chloride were purchased from Aldrich. *N*-Cbz-proline *p*-nitrophenyl ester and *N*^α,*N*^γ-di-Cbz-histidine were purchased from US Biochemical Corp. (Cleveland, OH, U.S.A.). Pyroglutamic pentachlorophenyl ester, trifluoroacetic acid, Na₂CO₃, chloramine-T, sodium metabisulphite, digitonin and buffer salts were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). *N*-Hydroxysuccinimidyl-(4'-azido)salicylate, *N*-hydroxysuccinimidyl-(4'-azido)benzoate and bincinchonic acid (BCA) protein assay reagents were purchased from Pierce (Rockford, IL, U.S.A.). CM-cellulose

Abbreviations used: TRH, thyrotropin-releasing hormone (pGlu-His-ProNH₂); TRH-ASA, pGlu-His-ProNH-(CH₂)₆-(4-azidosalicylamide); TRH-IASA, radioiodinated TRH-ASA; HAB, *N*-hexyl-4-azidobenzamide; Cbz, benzyloxycarbonyl; tBoc, *t*-butyloxycarbonyl.

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(CM52) was purchased from Whatman (Hillsboro, OR, U.S.A.). Silica gel and silica plates were obtained from J. T. Baker Inc. (Jackson, TN, U.S.A.). SepPak C₁₈ cartridges were obtained from Waters Associates (Milford, MA, U.S.A.).

Cell culture

Culture of GH₄C₁ cells has been described in detail previously (Tashjian *et al.*, 1968; Tashjian, 1979). Briefly, cells were grown in 100 mm plastic tissue culture dishes and fed every 3–4 days with Ham's F10 medium supplemented with 15% horse serum and 2.5% fetal bovine serum (F10⁺). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO₂/95% air. The cells used were routinely tested to ensure that cultures were free of mycoplasma contamination.

Protein determination

Cell protein was determined by the Pierce BCA assay (Smith *et al.*, 1985) using BSA (Sigma product A7906, lot 128F-0785) as a standard.

Synthesis of pGlu-His-ProNH-(CH₂)₆-(4'-azidosalicylamide) (TRH-ASA)

The synthesis of pGlu-His-ProNH-(CH₂)₆-(4-azidosalicylamide) is outlined as follows: (1) coupling of Cbz-Pro *p*-nitrophenyl ester to 1,6-diaminohexane; (2) reaction of Cbz-Pro-NH-(CH₂)₆-NH₂ with di-*t*-butyl dicarbonate; (3) H₂/Pd deprotection of Cbz-Pro-NH-(CH₂)₆-NH-tBoc and isobutyl chloroformate activation and coupling to N^α,N^ε-di-Cbz-His; (4) H₂/Pd deprotection of N^α,N^ε-di-Cbz-His-Pro-NH-(CH₂)₆-NH-tBoc and reaction with pyroglutamic pentachlorophenyl ester; (5) trifluoroacetic acid removal of the tBoc protective group and reaction with 4-azidosalicylic acid *N*-hydroxysuccinimide ester.

N-Hexyl-4-azidobenzamide (HAB) was synthesized by coupling 4-azidobenzoic acid *N*-hydroxysuccinimide ester and 1-aminohexane.

Details of the chemical procedures and purifications are available upon written request to the authors and as Supplementary Publication SUP 50167 (5 pages), which has been deposited at the British Library Document Supply Centre, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1992) **281**, 5.

Chemical confirmation and characterization of TRH-(CH₂)₆-NH-tBoc and TRH-ASA

The precursor TRH-(CH₂)₆-NH-tBoc was characterized by n.m.r. spectroscopy (Fig. 1) using a Varian VXR300 spectrometer. The ¹H-n.m.r. spectrum showed characteristic resonances (δ, p.p.m., in [²H₆]acetone) of the α-carbons (multiplets at 4.15, 4.35 and 4.70 p.p.m., 1 proton each), the imidazole ring protons (singlets, 7.00 and 7.62 p.p.m., 1 proton each), the tBoc methyl hydrogens (singlet, 1.35 p.p.m., 9 protons), and numerous aliphatic hydrogen peaks (1.25–3.60 p.p.m.). ¹³C-n.m.r. (in C²HCl₃) showed essential features of α-amido carbonyls (170–180 p.p.m.), imidazole carbons (130–140 p.p.m.), α-carbons (50–61 p.p.m.), tBoc methyl carbons (28 p.p.m.), and numerous aliphatic carbons (24–50 p.p.m.).

TRH-ASA was characterized by mass spectrometry (*m/z* = 623) using a VG ZAB-SE High Resolution Mass Spectrometer (VG Analytical, Manchester, U.K.) and by u.v. spectroscopy in water, with peaks at 272 and 304 nm with molar absorption coefficients of 8420 and 2840 M⁻¹·cm⁻¹ respectively. The 272 nm peak disappeared upon brief irradiation with u.v. light of 254 nm. The ¹³C-n.m.r. spectrum (in C²HCl₃, results not shown) was consistent with the predicted structure, with peaks for carbonyls (five peaks, 160–175 p.p.m.), imidazole and aryl

carbons (eight peaks, 110–135 p.p.m.), α-carbons (48–60 p.p.m.) and numerous aliphatic carbons (24–50 p.p.m.).

At a concentration of 25 nM, the material elicited an increase in the intracellular Ca²⁺ concentration of GH₄C₁ cells that was similar to that observed with authentic TRH (results not shown). In binding studies, TRH-ASA was shown to compete with [³H]*N*⁷-methyl-TRH for binding to GH₄C₁ cells, with an apparent dissociation constant of 25 nM (results not shown).

Radio-iodination of TRH-ASA

All procedures were performed under dim red light. TRH-ASA (29 nmol in 5 μl of acetonitrile) was added to 25 μl of 0.5 M-potassium phosphate, pH 7.5. This solution was added to Na¹²⁵I (5 mCi and 2 nmol, in 14 μl of NaOH, pH 8.0), and then chloramine-T (35 nmol in 8 μl of water) was added. After 60 s the reaction was quenched by the addition of Na₂S₂O₅ (169 nmol in 5 μl). Acetonitrile (40 μl) was then added, and the entire reaction mixture was applied to a reverse-phase (C₁₈) analytical h.p.l.c. column (Merck LiChrospher 100 RP-18; 5 μm). The column was washed with 25 mM-potassium citrate (pH 5.5) at 0.9 ml/min for 5 min, and then a gradient of 0–100% methanol over 140 min was applied at 0.9 ml/min. Radioactivity was eluted in two peaks centred at 116 min (40%) and 121–122 min (60%). The nature of these two peaks is discussed in the Results section.

Binding of iodinated TRH-ASA (TRH-IASA) to GH₄C₁ cells

GH₄C₁ cells were grown in 100 mm dishes as described above, harvested in HBSS buffer (see below) lacking Ca²⁺ (plus 600 μM-EDTA), and resuspended in HBSS buffer (118 mM-NaCl, 4.6 mM-KCl, 10 mM-D-glucose, 20 mM-Hepes, pH 7.4, and 0.4 mM-CaCl₂) at 37 °C. TRH-IASA in 25 mM-sodium phosphate buffer (pH 7.4) was added to the indicated final concentrations, and cells were incubated in a rotary incubator for 1 h at 37 °C. Aliquots of cells were then applied to glass fibre filters (Whatman GF/A) under suction, and rinsed with 3 × 5 ml of ice-cold 150 mM-NaCl. Retained TRH-IASA was evaluated by γ-radiation counting. Non-specific binding was determined by including TRH (20 μM) in the appropriate incubations.

Photoaffinity labelling of GH₄C₁ cells

Medium was aspirated from GH₄C₁ cells grown in either 100 mm dishes or 35 mm wells, and the cells were rinsed with warm (37 °C) HBSS buffer. Sufficient HBSS was added to cover the cells, and then TRH or other substances were added as described below for individual experiments. Finally (except where indicated), under dim red light, TRH-IASA was added in 0.5 ml of HBSS to a final concentration of approx. 3 nM. Cells were incubated in the dark at 37 °C for 30 min. Radioactive buffer was removed by aspiration, and dishes were washed with 2.5 ml of cold (4 °C) HBSS. All subsequent procedures were performed at 4 °C. Rinse buffer was removed by aspiration, and the cells were irradiated with u.v. light (two 15 W Sylvania G15T8 germicidal lamps at an average distance of 10 cm) for 2.5 min. Cells were scraped into cold buffer (20 mM-Mops, pH 7.4, 2 mM-MgCl₂, 1 mM-EGTA and 0.1% digitonin) and particulate material was precipitated by centrifugation. The membrane pellet was rinsed three times by resuspension/centrifugation in the same buffer.

SDS/PAGE and autoradiography

Labelled membrane pellets were dissolved in a small volume (approx. 100 μl/mg) of sample buffer (25 mM-Tris/HCl, pH 6.8, 10% SDS, 10% glycerol, 0.002% Bromophenol Blue and 10 mM-dithiothreitol). Some samples required 5 min of sonication for dissolution. Samples were incubated at 37 °C for 90 min, then *N*-ethylmaleimide was added to 25 mM, and samples were incubated for an additional 30 min. Samples were centrifuged and loaded

Fig. 1. (a) ^1H - and (b) ^{13}C -n.m.r. spectroscopy of $\text{TRH}-(\text{CH}_2)_6\text{-NH-tBoc}$

Spectra collection and peak assignments are described in the Experimental section. Large resonances at 2.03 p.p.m. (a) and 77 p.p.m. (b) are solvent resonances. The scale for the proton integration (continuous lines in a) can be estimated from the magnitude of each of the three α -carbon-attached single-proton resonances between 4.0 and 4.8 p.p.m.

(20–40 μl /lane) on to SDS/7.5%-PAGE gels (Laemmli, 1970). For autoradiography, stained and fixed gels were placed in a cassette with Kodak X-omat AR film and a Dupont Cronex enhancing screen, and then exposed at -70°C for 1–3 weeks.

RESULTS

Characterization of TRH-IASA

Preliminary studies indicated that ^{127}I -iodination of TRH-ASA using chloramine- τ resulted in the formation of a single u.v.-absorbing peak with mobility on reverse-phase h.p.l.c. just slightly less than that of non-iodinated TRH-ASA, followed closely by numerous peaks believed to be by-products of TRH-ASA and/or chloramine- τ . Following radio-iodination of TRH-ASA and h.p.l.c. purification as described in the Experimental section, two major peaks of radioactivity were eluted at $t = 116$ and 121 min. Fractions comprising these peaks were tested for specific binding to GH_4C_1 cells. The 116 min peak was found to contain most of the specific binding activity, and was concentrated into 25 mM-sodium phosphate, pH 7.4. The later peak,

Fig. 2. Saturation binding of TRH-IASA to GH_4C_1 cells (a) and Scatchard analysis of binding data (b)

This preparation of TRH-IASA was purified as described in the Experimental section, except that a 60 min gradient was used during the h.p.l.c. elution. Binding assays were performed as described in the Experimental section. Error bars represent the s.d.s observed with triplicate samples. Specific activity (220 Ci/mmol) and TRH-IASA concentrations were calculated from the Scatchard slope by assuming that the total number of specific sites (931 fmol/mg) would be the same as observed in parallel saturation binding assays using [$N^{\text{methyl-}}^3\text{H}$]TRH (results not shown). (a) $\circ$, Total binding; $\bullet$, non-specific binding; $\blacklozenge$, specific binding.

which also showed some specific binding, had much greater non-specific binding.

In an earlier preparation, TRH-IASA was purified as described above, except that the h.p.l.c. elution gradient was shortened to 1 h duration. Specific binding of TRH-IASA, from this preparation, to GH_4C_1 cells was shown to saturate at concentrations above 20 nM (Fig. 2). Parallel binding studies using [^3H]N $^{\text{methyl-}}$ TRH (specific radioactivity 56 Ci/mmol) showed that these cells possessed approx. 930 fmol/mg of binding sites. This value was used to calculate a specific radioactivity of 220 Ci/mmol and a K_d of 11 nM for this preparation of TRH-IASA. TRH-IASA prepared using a 140 min gradient, as described in the Experimental section, had a specific radioactivity (1560 Ci/mmol) close to the theoretical value (2200 Ci/mmol),

Fig. 3. Binding of TRH-IASA to GH₄C₁ and GH₁₂C₁ cells incubated without or with competing additives

Cells were incubated with TRH-IASA, irradiated, washed and solubilized as described in the Experimental section, with a final concentration of approx. 1.3 mg of cell protein/ml. (a) Radioactivity incorporated into aliquots of solubilized membranes was measured by γ -radiation counting, and is reported as c.p.m./ μ l. These results are consistent with observations from a duplicate experiment. The first bar in each panel (control) gives the TRH-IASA bound in the absence of any competing ligand. The concentrations of all competitors was 50 μ M, except for TRH (8 μ M). Abbreviation: CDZ, chlordiazepoxide. (b) Aliquots (approx. 30 μ g of protein) from (a) were applied to SDS/PAGE followed by autoradiography. All lanes represent samples from GH₄C₁ cells, except for the four lanes specifically indicated from GH₁₂C₁ cells. Loading of the gel was not uniform due to high viscosity of these samples.

and bound specifically to GH₄C₁ cells with a K_d of 8 nM (results not shown), indicating that the longer h.p.l.c. gradient effectively separated TRH-IASA from TRH-ASA.

Photoaffinity labelling of the TRH receptor

GH₄C₁ and GH₁₂C₁ cells were incubated with TRH-IASA in the presence of various competing ligands, then photolysed and solubilized as described in the Experimental section. Aliquots were removed for direct γ -radiation counting (Fig. 3a), and samples were applied to SDS/PAGE and autoradiography (Fig. 3b). As shown in Fig. 3(a), TRH-IASA labelling was completely blocked by TRH, largely inhibited by 50 μ M-chlordiazepoxide (a competitive TRH receptor antagonist, K_d 10 μ M; Simasko &

Fig. 4. Effects of duration of incubation, photolysis period and TRH-IASA concentration on labelling of GH₄C₁ cells

GH₄C₁ cells were labelled with TRH-IASA as described in the Experimental section, with the following modifications. In lanes 1–3, the duration of the incubation with TRH-IASA at 37°C was for 5 min, 40 min or 2 h respectively, and equal amounts of c.p.m. were applied to each lane for SDS/PAGE and autoradiography. In lane 4, the cells were incubated for 40 min with 3 nM-TRH-IASA and rinsed, and then exposed to u.v. light for only 5 s. In lanes 5–10, the concentration of TRH-IASA was varied from 0.55 to 28.6 nM, as indicated, and equal volumes of solubilized membranes were applied to SDS/PAGE and autoradiography.

Horita, 1984; Drummond, 1985; Gershengorn & Paul, 1986), and weakly inhibited by the weak agonists (Hinkle *et al.*, 1974) pGlu-His-GlyNH₂ (K_d > 30 μ M) and pGlu-His-ProOH (K_d 13 μ M). To confirm that no significant binding was occurring due to the *N*-hexyl-4-azidosalicylamide tail of TRH-IASA, the analogue HAB was examined. HAB did not compete with TRH-IASA for binding to GH₄C₁ cells (Fig. 3a), confirming that labelling was due only to the TRH-like portion of TRH-IASA. No evidence for specific binding of TRH-IASA was seen after incubation of GH₁₂C₁ cells with TRH-IASA; these cells lack functional TRH receptors (Tashjian, 1979).

SDS/PAGE and autoradiography of these samples (Fig. 3b) showed predominant labelling of a single diffuse band of molecular mass 71–91 kDa (centred at 76 kDa). Labelling was completely absent from cells incubated with TRH-IASA plus TRH, and was not observed using GH₁₂C₁ cells under any conditions. Samples used in this experiment had not been sonicated and were highly viscous, accounting for the uneven loading observed between lanes.

Other minor bands were observed on this autoradiogram, and in order to establish which of the labelled bands might represent proteins which bind TRH-IASA specifically, additional experiments were performed.

Effect of duration of incubation on the labelling pattern

GH₄C₁ cells were labelled with TRH-IASA as described in the Experimental section, except that the duration of the incubation was varied from 5 min to 2 h. Total counts incorporated increased for up to 30 min (results not shown), but when samples were applied to SDS/PAGE and autoradiography (equal c.p.m./lane), no change was observed in the overall labelling pattern (Fig. 4, lanes 1–3, and results not shown).

Following a 40 min incubation of GH₄C₁ cells with 3 nM-TRH-IASA, cells were rinsed and then the duration of the exposure to u.v. light was varied between 5 s and 5 min. Short

exposures were found to label only the 76 kDa band (Fig. 4, lane 4).

Dependence of labelling on TRH-IASA concentration

Labelling of GH₄C₁ cells was performed as described in the Experimental section, except the concentration of TRH-IASA was varied between 0.6 and 29 nM. As shown in Fig. 4 (lanes 5–10), at low TRH-IASA concentrations only the diffuse band centred at 76 kDa was strongly labelled. At higher concentrations, other bands appeared at approx. 37, 49, 54, 120 and 170 kDa; the intensity of these bands was never more than about 20% of the intensity of the 76 kDa band (Fig. 4, lane 10). The nature and specificity of these bands is currently uncertain; they may originate from non-specific associations during the electrophoretic separation.

DISCUSSION

A facile synthetic route for preparation of TRH-ASA was decided on after surveying several published syntheses of TRH and analogues (Goebel *et al.*, 1981; Grim *et al.*, 1981; Labroo *et al.*, 1983; Szirtes *et al.*, 1984, 1986). The synthesis was facilitated by the availability of pre-activated proline and pyroglutamyl esters, and no unusual difficulties were encountered during the synthetic procedures. During iodination of TRH-ASA, the ratio of chloramine-T to TRH-ASA (approx. 1.2:1 optimal) was found to be a critical factor, with higher proportions of chloramine-T yielding large amounts of inactive by-products.

Our efforts to photoaffinity label TRH receptors in either particulate or solubilized membrane preparations [prepared according to Hinkle & Kinsella (1984) and Phillips & Hinkle (1989) respectively] from GH₄C₁ cells were frustrated by high non-specific binding of TRH-IASA to these preparations. We suspect that the hydrophobic 4-azido-*N*-hexyl-5-iodosalicylamide tail of TRH-IASA, combined with the relatively polar peptide portion, results in an amphipathic molecule which, like a detergent, is readily incorporated into exposed membranes or micellar structures. Low non-specific incorporation of TRH-IASA was achieved only with intact GH₄C₁ cells (Fig. 3a), using a protocol which included several mild detergent washes of the labelled cell membranes.

The 2.5 min u.v. irradiation used for most of the experiments shown in this study was found in a later experiment (Fig. 4, lane 4) to be excessive. The original 2.5 min exposure was chosen because many prior studies using other arylazide-based photoaffinity labels (Hazum, 1983, and references therein) utilized either high-intensity lamps for short intervals (e.g. Lavin *et al.*, 1982) or low-intensity lamps for extended times (e.g. Moseley *et al.*, 1987). The value of a shorter photolysis interval for producing more specific labelling was clearly demonstrated in our study.

Standard procedures for preparing samples for SDS/PAGE (e.g. briefly boiling samples in a 1% SDS buffer; Laemmli, 1970) were found to cause aggregation of labelled proteins and subsequent failure to penetrate polyacrylamide gels. This is a common problem with integral membrane proteins (Hames & Rickwood, 1981), and was circumvented by using a high-SDS sample buffer according to Shorr *et al.* (1982). The 56 °C incubation used in that study was found to cause aggregation of the TRH-IASA-labelled proteins, so the temperature was further decreased to 37 °C in our experiments.

The primary structure of the TRH receptor from thyrotropin-producing mouse pituitary tumour tissue has recently been reported (Straub *et al.*, 1990), showing this receptor to share sequence similarity with the growing family of G-protein-linked plasma membrane receptors (Dohlman *et al.*, 1987; Taylor, 1990). The mouse pituitary thyrotroph receptor has two potential

N-linked glycosylation sites near its *N*-terminus. Furthermore, it has been shown that in solubilized GH₄C₁ membranes the TRH-binding activity can be reversibly bound to wheat-germ-agglutinin-agarose (Phillips & Hinkle, 1989), indicating that the rat receptor is a glycoprotein. The glycoprotein nature of the TRH receptor might therefore account for the diffuse character of the major TRH-IASA-labelled band in SDS/PAGE. Several attempts to deglycosylate the 76 kDa protein using either peptide-*N*-glycanase F (Gerard, 1990) or trifluoromethanesulphonic acid (Sojar & Bahl, 1987) have not been successful, owing to enzyme/detergent incompatibilities and to the tendency for the 76 kDa protein to form high-molecular-mass aggregates during incubation.

We believe that our TRH-IASA reagent labels specifically the TRH receptor on GH₄C₁ cells for the following reasons: (1) TRH-IASA has appropriately high-affinity binding to receptor-positive GH₄C₁ cells and essentially no binding to GH₁2C₁ pituitary cells, which lack functional TRH receptors; (2) binding to GH₄C₁ cells is selectively inhibited by TRH, TRH agonists and chlordiazepoxide, but not by HAB; and (3) SDS/PAGE analysis of proteins covalently labelled by TRH-IASA revealed a highly predominant band which behaves as a glycoprotein and has a molecular mass which is compatible with the core protein molecular mass of 44.5 kDa for the mouse thyrotroph TRH receptor (Straub *et al.*, 1990) plus the anticipated additional mass contributed by the *N*-linked carbohydrate. However, it is not possible to conclude with complete certainty that the 76 kDa band represents the rat pituitary lactotroph TRH receptor. It remains possible that, while the TRH-like portion of TRH-IASA may attach in the TRH binding site, the extended tail of TRH-IASA, to which the photolabile azide group is attached, might bind with high affinity to a closely associated molecule. This possibility can be addressed when sufficient quantities of the 76 kDa protein are available to allow peptide sequencing, or by immunoprecipitation of the 76 kDa protein with TRH-receptor-specific antibodies. Meanwhile, the size of the labelled protein lies well within the range observed for other members of this receptor family studied by photoaffinity techniques [cf. β -adrenergic receptors, 38–65 kDa (Lavin *et al.*, 1982); calcitonin receptor, 85 kDa (Moseley *et al.*, 1987); cholecystokinin receptor, 85–95 kDa (Duong *et al.*, 1989)].

In conclusion, we have prepared a photoaffinity analogue of TRH, TRH-IASA, which binds with high affinity and specificity to rat pituitary GH₄C₁ cells. At concentrations below 3 nM, TRH-IASA specifically labels a target protein which migrates in SDS/PAGE as a diffuse band (71–91 kDa) centred at 76 kDa.

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