Comparison of the signalling properties of the long and short isoforms of the rat thyrotropin-releasing-hormone receptor following expression in Rat 1 fibroblasts

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cDNA species encoding either the long or the short isoforms of the rat thyrotropin-releasing-hormone (TRH) receptor were expressed stably in Rat 1 fibroblasts, and clones expressing specific binding of [^{3}H]TRH were detected and expanded. Clones expressing each of these receptors at levels up to 1 pmol/mg of membrane protein were selected for analysis. Reverse-transcriptase PCR on RNA isolated from these clones confirmed that each clone expressed only mRNA corresponding to the expected splice variant. Both receptor splice variants bound [^{3}H]TRH with a K_d of some 80 nM when binding assays were performed in the presence of guanosine 5′-[$\beta\gamma$-imido]-triphosphate. In the presence of TRH, both receptor subtypes were able to cause stimulation of inositol phosphate generation in a pertussis-toxin-insensitive manner with similar EC_{50} values and to stimulate the mobilization of intracellular Ca^{2+}, but, despite reports that TRH receptors can also interact with the G-proteins G_s and G_i2, neither receptor splice variant was able to modulate adenylate cyclase activity in either a positive or a negative manner. These data indicate that the long and short isoforms of the rat TRH receptor have similar affinities for TRH and display similar abilities to interact with the G_q-like G-proteins, but show no ability to regulate adenylate cyclase, at least when expressed in this genetic background.

INTRODUCTION

The isolation of cDNA clones encoding mouse [1] and rat [2,3] versions of the thyrotropin-releasing-hormone (TRH) receptor confirmed these to be single-polypeptide putative seven-transmembrane-element receptors, as anticipated for G-protein-coupled receptors. Although a series of reports have indicated that these receptors couple in a pertussis-toxin-insensitive fashion to the stimulation of inositol phosphate and diacylglycerol production [4,5], and indeed have been shown directly to do so by interaction with G_q and G_{11} [4,5], a number of reports in which TRH responses have been analysed in GH3 and other related cell lines of pituitary origin have indicated both a direct interaction of the TRH receptor with G_s to cause activation of adenylate cyclase [6] and a TRH-mediated activation of G_i2 and thence an increase in Ca^{2+} flux through L-type Ca^{2+} channels [7], although it is uncertain whether the TRH receptor directly interacts with G_i2 or whether this occurs subsequent to stimulation of protein kinase C [7].

A second isoform of the rat TRH receptor was subsequently cloned from a GH3-cell library [8]. This short isoform of the receptor, which appeared to be derived from the same gene as the long isoform by differential splicing mechanisms [8], was also shown to be expressed in the pituitary. The existence of two splice variants of the rat TRH receptor thus raises the possibility that the range of effects reported in response to TRH treatment of pituitary-derived GH-cell lines may reflect the activation of two pharmacologically similar, but genetically distinct, receptor species. To examine this question, in this study we have expressed cDNA species encoding each of the long and the short isoforms of the rat TRH receptor in Rat 1 fibroblasts and then examined the signalling properties of the two isoforms.

MATERIALS AND METHODS

Materials

All materials for tissue culture were from Life Technologies Inc. [^{3}H]TRH (45–56 Ci/mmol), [α-^{32}P]ATP, cyclic [^{3}H]AMP and *myo*-[^{3}H]inositol were from Amersham International. Pertussis toxin was from Porton Products.

Generation and isolation of clones of Rat-1 fibroblasts expressing long and short isoforms of the rat TRH receptor

A full-length rat TRH receptor (long isoform) cDNA (2.2 kb) was subcloned into the eukaryotic expression vector pcDNA1 as described previously [5]. A full-length rat TRH receptor (short isoform) [8] was obtained in pBluescript SK$^-$ from Dr. Pilar de la Pena, University of Oviedo, Spain. This was subcloned into the eukaryotic expression vector pCMV5 between the *Xba*1 and *Bam*H1 sites of the multiple cloning region. Both of these constructs were transfected into monolayer cultures of Rat-1 fibroblasts along with the plasmid pSV2neo (Invitrogen) by using Lipofectin reagent (Life Technologies Inc.), as suggested by the manufacturer, in serum-free Dulbecco's modified Eagle's medium (DMEM). After 16 h medium was replaced with serum containing DMEM, and 48 h later geneticin sulphate (750 μg/ml) (Life Technologies Inc.) was added to initiate selection. Resulting geneticin-resistant clones were picked, expanded and assayed for the presence of the TRH receptor (see below).

Cell growth

Parental Rat-1 fibroblasts and clones derived from these cells expressing the TRH receptor isoforms were grown in tissue

Abbreviations used: TRH, thyrotropin-releasing hormone (thyroliberin); DMEM, Dulbecco's modified Eagle's medium; NP40, Nonidet P40; RT-PCR, reverse-transcriptase PCR; p[NH]ppG, guanosine 5′-[$\beta\gamma$-imido]triphosphate; ERK-1, extracellular regulated kinase-1 [also called 44 kDa MAP (mitogen-activated protein) kinase]; $[Ca^{2+}]_i$, intracellular concentration of Ca^{2+}.

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culture in DMEM containing 5% newborn-calf serum. The receptor-expressing clones were further maintained in the presence of 750 μg/ml geneticin sulphate. Before confluency they were either split 1:10 into fresh tissue-culture flasks or harvested. Membrane fractions were prepared from cell pastes which had been stored at −80 °C after harvest essentially as described in [9]. Frozen cell pellets were suspended in 5 ml of 10 mM Tris/HCl/0.1 mM EDTA, pH 7.5 (buffer A), and cells were ruptured with 25 strokes of a hand-held Teflon-on-glass homogenizer. The resulting homogenate was centrifuged at 500 *g* for 10 min in a Beckman L5-50B centrifuge with a Ti 50 rotor, to remove unbroken cells and nuclei. The supernatant fraction from this treatment was then centrifuged at 40000 *g* for 10 min, and the pellet from this treatment was washed and resuspended in 10 ml of buffer A. After a second centrifugation at 48000 *g* for 10 min, the membrane pellet was resuspended in buffer A to a final protein concentration of 1–3 mg/ml and stored at −80 °C until required.

Production of antisera and immunoblotting

Antiserum CS was produced by a New Zealand White rabbit after immunization with a glutaraldehyde conjugate of keyhole-limpet haemocyanin (Calbiochem) and a synthetic peptide, RMHLRQYELL, which corresponds to the C-terminal decapeptide of all forms of the α subunit of G_s. The specificity of this antiserum for $G_s\alpha$ has previously been demonstrated [10]. Immunoblotting with this antiserum was performed as previously described [11]. Antiserum CQ [12] was generated against a synthetic peptide (QLNLKEYNLV) which represents the C-terminal decapeptide which is conserved between $G_q\alpha$ and $G_{11}\alpha$ [13]. As such, this antiserum cannot distinguish between these two polypeptides unless they are resolved by electrophoretic techniques. This antiserum has been shown directly, however, to identify both polypeptides equally [14]. Antiserum SG was produced against the C-terminal decapeptide of the α subunit of rod transducin (KENLKDCGLF) [15]. This antiserum identifies both $G_i1\alpha$ and $G_i2\alpha$ as well as transducin α. The specificities and characterization of these antisera have been fully described. Molecular-mass determinations were based on pre-stained molecular-mass markers (Bethesda Research Laboratories). SDS/PAGE [10% (w/v) acrylamide gels] was carried out overnight at 60 V.

After SDS/PAGE, proteins were transferred to nitrocellulose (Schleicher and Schuell) and blocked for 2 h in 5% gelatin in PBS, pH 7.5. Primary antisera were added in 1% gelatin in PBS containing 0.2% Nonidet P40 (NP40) and incubated for at least 2 h. The primary antiserum was then removed and the blot washed extensively with PBS containing 0.2% NP40. Secondary antiserum (donkey anti-rabbit IgG coupled to horseradish peroxidase; Scottish Antibody Production Unit, Wishaw, Scotland, U.K.) was added (1:200 dilution in 1% gelatin in PBS containing 0.2% NP40) and incubated with the nitrocellulose for 2 h. The antiserum was then removed and, after extensive washing of the blot with PBS containing 0.2% NP40 and finally with PBS alone, the blot was developed by using *o*-dianisidine hydrochloride (Sigma) as the substrate for horseradish peroxidase as previously described [11].

TRH regulation of inositol phosphate production

Cells were seeded in 24-well plates and labelled to isotopic equilibrium with [^{3}H]inositol (1 μCi/ml) in inositol-free DMEM containing 1% dialysed newborn-calf serum for 48 hours. The cells were used when confluent and quiescent.

On the day of experiments, the labelling medium was removed and the cells were washed twice with 0.5 ml of Hanks buffered saline, pH 7.4, containing 1% (w/v) BSA and 10 mM glucose (HBG). The cells were incubated for 10 min with HBG containing 10 mM LiCl (HGB/LiCl) and stimulation was carried out with agonist in HBG/LiCl for 20 min. All incubations were performed at 37 °C. Reactions were terminated by addition of 0.5 ml of ice-cold methanol. The cells in each well were then scraped and transferred to vials. Chloroform was added to a ratio of 1:2 (chloroform/methanol) and the samples extracted overnight. The phases were split by the addition of chloroform and water to final proportions of 1:1:0.9 (chloroform/methanol/water) and the upper phase was taken for analysis of total inositol phosphates. Total inositol phosphates were analysed by batch chromatography on Dowex-1 (formate form) as previously described [16].

Adenylate cyclase assays

These were performed as described by Milligan et al. [17]. Radiolabelled cyclic AMP and ATP were separated by the method of Salomon et al. [18].

Binding experiments with [^{3}H]TRH

These were performed routinely with ~ 10 nM [^{3}H]TRH at 30 °C for 30 min in 20 mM Tris/HCl (pH 7.5)/50 mM sucrose/20 mM $MgCl_2$/100 μM guanosine 5′-[$\beta\gamma$-imido]triphosphate (p[NH]ppG) (buffer B) in the absence and presence of 100 μM TRH to define maximal and non-specific binding respectively. Specific binding, defined as above, represented ~ 80% of the total binding of [^{3}H]TRH. In experiments designed to assess the maximal binding capacity of membranes of transfected cells for this ligand, the specific radioactivity of a single concentration (~ 10 nM) of [^{3}H]TRH was varied by addition of TRH, and measured specific binding was subsequently corrected on this basis. All binding experiments were terminated by rapid filtration through Whatman GF/C filters, followed by three washes (each 5 ml) with ice-cold buffer B. In several cases, equivalent binding assays were performed in the absence of p[NH]ppG.

Intracellular Ca^{2+} ($[Ca^{2+}]_i$) measurements

$[Ca^{2+}]_i$ measurements were carried out as previously described [19]. In brief, single cells grown on glass coverslips were loaded in buffer A with fura-2 AM (4 μM final concn.) for 30 min at 37 °C in a 5%-CO_2 humidified incubator. Washed coverslips were inserted into a coverslip chamber and transferred into a heated stage (37 °C) of an inverted epifluorescence microscope (see below). Dynamic video imaging was carried out with the MagiCal hardware and Tardis software provided by Joyce Loebl, Gateshead, U.K. A Nikon Diaphot microscope with a ×40 quartz oil-immersion objective, operated in epifluorescence mode, was used to image the cells. Fluorescence excitation shifts occur when fura-2 binds Ca^{2+}, i.e. the excitation efficiency increases at 340 nm and decreases at 380 nm. Ratios of values obtained at 340 nm/380 nm therefore represent changes in $[Ca^{2+}]_i$. Fluorescent images were obtained by exposing cells to 340 and 380 nm light. The viewed images were focused on to the face of an intensified charge-coupled device camera (Photonic Sciences, Robertsbridge, East Sussex, U.K.). These images were held in memory for processing; 340/380 nm ratios were subsequently calculated.

Reverse-transcriptase PCR (RT-PCR)

The overall RT-PCR procedure has been described previously [20].

RNA extractions

Total RNA was extracted by the acid phenol/guanidinium thiocyanate method of Chomczynski and Sacchi [21]. Purity and quantification were assessed by A_{260}/A_{280} ratios.

Reverse transcription

RNA was precipitated with NaCl/ethanol and redissolved in 5 μl of water. Samples (1 μg) were denatured and reverse-transcribed by using a first-strand cDNA synthesis kit (Pharmacia). Incubation was carried out at 37 °C for 1 h and stopped by incubation at 80 °C for 10 min. Reaction mixtures were not extracted and were stored at −20 °C before amplification.

PCR amplification

PCR reactions were carried out with the following 24-mers (synthesized on an Applied Biosystems synthesizer):

TRH-sense 5′ CAACCTCATGTCTCAGAAGTTTCG

TRH-antisense 5′ AATGAAGACCTTCGATCAGTTGG

Amplifications for the TRH receptor isoforms were performed over 35 cycles of PCR carried out in the presence of 25 pmol of sense and antisense primers, dNTPs (0.2 mM of each dATP, dCTP, dGTP, dTTP), 6 mm $MgCl_2$ and *Taq* polymerase (2.5 units) (Promega) in a final volume of 100 μl. Amplifications were carried out in a Hybrid Omnigene thermocycler. Cycling conditions were 95 °C/30 s, 48 °C/1 min, 72 °C/1 min, followed by a final extension at 72 °C for 2 min.

Reaction products were separated by 2% (w/v) agarose-gel electrophoresis. No signals were observed in samples that were amplified without prior reverse transcription.

Regulation of activity/mobility of ERK-1 (extracellularly regulated kinase 1)

The activation of ERK-1 was determined by an electrophoretic-mobility-shift assay [22–24]. Cells were stimulated with the appropriate ligand for 5 min (see the Results section for details) after maintenance in serum-free medium for 48 h and subsequently lysed at 4 °C in a buffer containing 25 mM Tris/HCl, 40 mM *p*-nitrophenol, 25 mM NaCl, pH 7.5, 10% (v/v) ethylene glycol, 10 μM dithiothreitol, 0.2% (w/v) NP40, 1 μg/ml aprotinin, 1 mM sodium vanadate, 3.5 μg/ml pepstatin A and 200 μM phenylmethanesulphonyl fluoride at pH 7.5. After centrifugation of the lysed samples in a Microfuge (13000 rev./min, 5 min), SDS/PAGE loading buffer was added to a sample of the supernatant and applied to SDS/PAGE gel (10% acrylamide containing 6M urea) after heating of the sample in a boiling-water bath for 5 min. Samples were transferred from the gel to nitrocellulose and immunoblotted with an anti-peptide antiserum raised against amino acids 325–345 of ERK-1, which is also called p44 MAP kinase (kindly given by Dr. N. G. Anderson, Hannah Research Institute, Ayr, Scotland). These gel conditions provide excellent 'gel shift' of this polypeptide upon ligand regulation, a feature which is synonymous with its phosphorylation and activation [22–24].

Data analysis

Analysis of the TRH-receptor self-displacement binding data and inositol phosphate generation curves was performed by using the Kaleidagraph curve-fitting package driven by an Apple Macintosh computer.

RESULTS

Rat-1 fibroblasts were stably transfected with either plasmid pcDNA1, containing the long isoform of the rat TRH receptor [5] (10 μg), or plasmid pCMV5, into which the short isoform of the rat TRH receptor [8] had been ligated (10 μg). In both cases plasmid pSV2neo (1 μg) was co-transfected along with the receptor-containing plasmids to allow for selection of clones

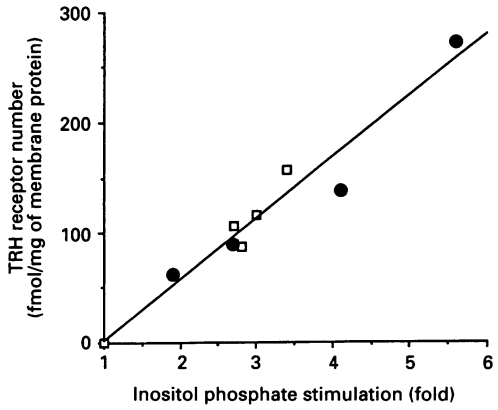

Figure 1 Correlation between TRH-receptor expression levels and TRH-stimulated inositol phosphate generation for Rat 1 fibroblasts expressing long and short TRH receptor isoforms

The specific binding of a single concentration of [^{3}H]TRH (10 nM) defined by the absence and presence of 10 μM TRH in the presence of 100 μM p[NH]ppG was measured as was the ability of 10 μM TRH to stimulate production of inositol phosphates in a 10 min assay as described in the Materials and methods section in a number of clones of Rat-1 fibroblasts transfected to express either the short (●) or long (□) isoforms of the Rat TRH receptor. The receptor-binding data have not been corrected for receptor occupancy. Clones which were transfected with receptor-containing plasmids and which became resistant to geneticin sulphate, but failed to display specific binding of [^{3}H]TRH, also failed to accumulate inositol phosphates. Data are taken from the following clones: long isoform, clones 25, 32, 34 and 42; short isoform, clones 1, 4, 5 and 7.

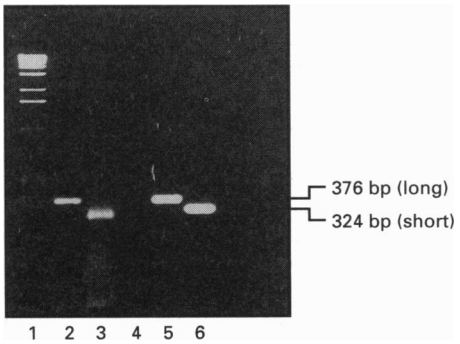

Figure 2 Specific detection of mRNAs encoding either the long or short isoforms of the rat TRH receptor

RNA isolated from either clone 32 (transfected with long-isoform cDNA) (lane 2) or clone 1 (short-isoform) (lane 3) was reverse-transcribed and PCR was performed with the primers described in the Materials and methods section. Positive controls were provided by the long (lane 5) and short (lane 6) cDNA species, and a negative control (lane 4) was obtained by omission of template. A 1 kb ladder provided size markers (lane 1).

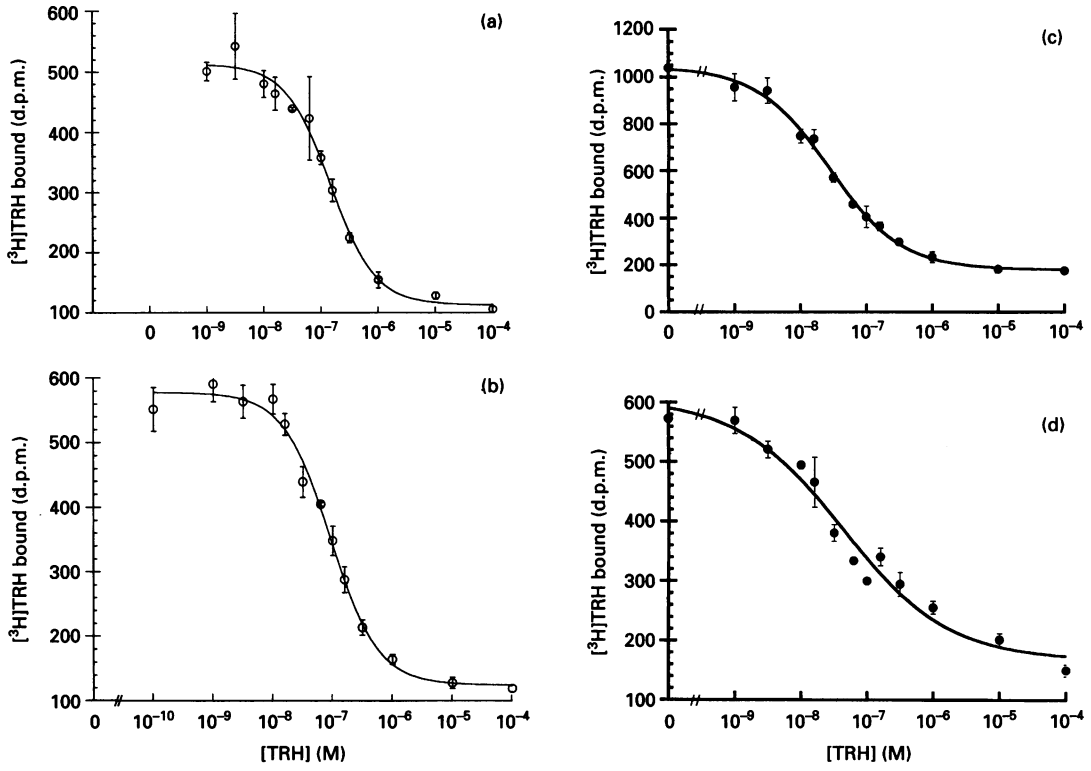

Figure 3 [^{3}H]TRH-binding characteristics of long and short splice variants of the rat TRH receptor

The ability of various concentrations of TRH to compete with [^{3}H]TRH for binding to the TRH-receptor isoforms expressed in membranes (50 μg) of clones 1 (short form) and 32 (long form) was assessed in the presence (**a**, **b**) or absence (**c**, **d**) of 100 μM p[NH]ppG as described in the Materials and methods section. (**a**) Clone 1. In the example displayed, [^{3}H]TRH was 9.9 nM and the estimated IC_{50} for TRH was 150 nM; h was 1.11. Application of the formalisms of DeBlasi et al. [32] therefore allows estimation of a K_d for TRH of 140 nM. (**b**) Clone 32. In the example displayed, [^{3}H]TRH was 10.7 nM and the estimated IC_{50} for TRH was 93 nM; h was 1.09. These data provide an estimate of K_d for TRH of 82 nM. (**c**) Clone 1. In the example displayed, [^{3}H]TRH was 11.0 nM and the estimated IC_{50} for TRH was 27 nM; h was 0.78. A h value of less than 1 implies the likely existence of more than one detected affinity state for TRH and precludes use of the formalisms of DeBlasi et al. [32]. Application of a two-component fit model allowed estimation of the K_d of the high-affinity site for TRH of 8.6 nM. (**d**) Clone 32. In the example displayed, [^{3}H]TRH was 11.0 nM and the estimated IC_{50} for TRH was 38 nM; h was 0.6. Application of a two-component fit model allowed estimation of the K_d of the high-affinity site for TRH of 10.5 nM.

expressing resistance to geneticin sulphate (750 μg/ml). Resistant colonies were selected and expanded and expression of the TRH-receptor isoforms initially screened for by the ability of TRH (10 μM) to promote generation of [^{3}H]inositol phosphates after labelling of cells with *myo*-[^{3}H]inositol (1 μCi/ml). A number of clones were positive in such assays (Figure 1), and these positive clones were further defined by analysis of the specific binding (defined by the absence and presence of 10 μM TRH) to membranes of these cells of (in these preliminary studies) a single concentration of [^{3}H]TRH (10 nM) in the presence of the poorly hydrolysed analogue of GTP, p[NH]ppG (100 μM). These clones exhibited a range of levels of TRH-receptor binding (Figure 1). The correlation between receptor levels and fold stimulation of inositol phosphate levels above basal was high when clones transfected with plasmids incorporating either the long or short splice variants was examined ($r = 0.94$).

Clone 32, transfected to express the long isoform of the receptor, and clone 1, transfected with the short-isoform cDNA, were selected for detailed study. To confirm solely the expression of the expected TRH-receptor isoforms in these cells, RNA was isolated and subjected to reverse transcription followed by PCR (RT-PCR) by using a primer pair which straddles the splice variation site in the TRH-receptor gene. A single PCR product of 376 bp was obtained from RNA isolated from clone 32, and a single PCR product of 324 bases from RNA isolated from clone 1 (Figure 2). These were the expected products from the long and short isoforms respectively. Confirmation of this was obtained from PCR reactions performed with the two cDNA species ligated into the plasmids used for their expression (Figure 2). No appropriate product was obtained either when template was excluded from the PCR reaction (Figure 2) or when RNA isolated from parental Rat-1 fibroblasts was subjected to RT-PCR with these primers (results not shown).

Maximal levels and the affinity of TRH binding to the long and short isoforms of the receptor expressed in membranes of clones 32 and 1 was examined by analysis of competition curves between [^{3}H]TRH (approx. 10 nM) and various concentrations of TRH in the presence of 100 μM p[NH]ppG (Figures 3a and 3b). Analysis of such data indicated that the long isoform of the receptor was expressed at 750 ± 30 fmol/mg of membrane protein in clone 32, and the short isoform of the receptor was expressed at 860 ± 170 fmol/mg of membrane protein in clone 1. The estimated K_d for the binding of TRH to these receptors in these assay conditions was 85 ± 3 nM for the long isoform and 71 ± 34 nM for the short isoform (mean $\pm$ S.E.M.; $n = 3$ in each case). These values were not significantly different ($P = 0.76$). These values must, however, be interpreted with caution, as binding parameters based on analysis of binding of ^{3}H-labelled agonist ligands can only be expected to provide approximate values. Similar binding experiments were also performed in the

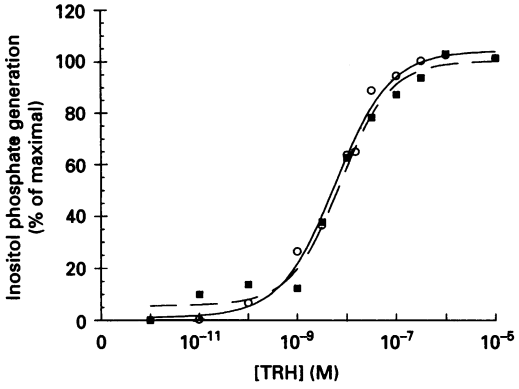

Figure 4 Comparison of the ability of TRH to stimulate inositol phosphate generation in Rat 1-fibroblast clones expressing either the long or the short isoforms of the rat TRH receptor

The ability of various concentrations of TRH to stimulate production of inositol phosphates was assessed in cells of clones 1 (short isoform) (○) and 32 (long isoform) (■) as described in the Materials and methods section. Results are presented as percentage of maximal effect (defined as the stimulation obtained with 10 μM TRH).

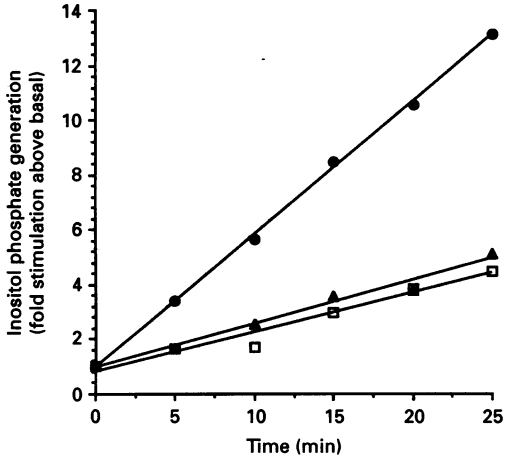

Figure 5 Inositol phosphate production by either the long or the short TRH-receptor isoforms: time-course studies

The ability of TRH (10 μM) to stimulate inositol phosphate production was assessed as described in the Materials and methods section at various time points over a period of 25 min in clones 1 (●) and 7 (▲) (both short isoform) and clone 32 (□) (long isoform). The higher fold stimulation of inositol phosphate generation in clone 1 in response to TRH in these studies was largely a reflection of lower levels of inositol phosphates being present in incubations performed in the absence of TRH.

absence of p[NH]ppG (Figures 3c and 3d). In such experiments, the Hill coefficient (slope) of the displacement curve of specific [^{3}H]TRH binding by TRH was substantially less than 1. Analyses of such data by a two-site fit model indicated that $57 \pm 8\%$ (mean $\pm$ S.D., $n = 3$) of the long isoform of the receptor was in the high-affinity state in these experiments. This was not significantly different ($P = 0.31$) from the short isoform, in which some $48 \pm 11\%$ (mean $\pm$ S.D., $n = 3$) of the receptors were estimated to be in the same high-affinity state. The estimated K_d for the binding of TRH to the high-affinity state of the long isoform of the receptor was 8.2 ± 4.0 nM (mean $\pm$ S.D., $n = 3$), and for the short isoform it was 12.1 ± 3.8 nM (mean $\pm$ S.D., $n = 3$). These values were not significantly different from one another ($P = 0.29$). The characteristics of the low-affinity state of the receptor for TRH for each isoform were not amenable to analysis by this means, as the estimated errors were at least as great as the values obtained (results not shown).

Examination of the ability of TRH to stimulate inositol phosphate production in *myo*-[^{3}H]inositol-labelled clone 32 and clone 1 cells in the presence of LiCl (10 mM) demonstrated that the EC_{50} for TRH was highly similar for the two receptors (7.5 ± 1.7 nM for long isoform, 6.0 ± 0.9 nM for short isoform) (Figure 4). This was unaffected by pretreatment of the cells with pertussis toxin (25 ng/ml for 16 h), conditions able to cause ADP-ribosylation of essentially the entire population of pertussis-toxin-sensitive G-proteins in these cells (results not shown). Examination of time courses of inositol phosphate generation by the two TRH-receptor splice variants was assessed over a period of 25 min. In both clone 32 (long isoform) and clone 1 (short isoform) the rate of inositol phosphate generation was linear over this time period. As clone 1 produced a larger fold stimulation of inositol phosphate generation than clone 32, a second clone (clone 7) expressing the short form of the receptor was also tested in parallel. This clone also displayed linear kinetics of inositol phosphate production in response to TRH (Figure 5).

TRH (1 μM) increased $[Ca^{2+}]_i$ in both clone 32 and clone 1. In clone 32 the peak rise in $[Ca^{2+}]_i$ (estimated to be from 13 ± 5 nM in the absence of agonist to 210 ± 18 nM in the presence of TRH) was somewhat lower than that achieved by clone 1 (basal 17 ± 5 nM, to 340 ± 22 in the presence of TRH) (mean $\pm$ S.E.M.; $n = 3$ in each case). The decay back towards resting $[Ca^{2+}]_i$ levels also occurred over a longer period in clone 1 than in clone 32 (Figure 6).

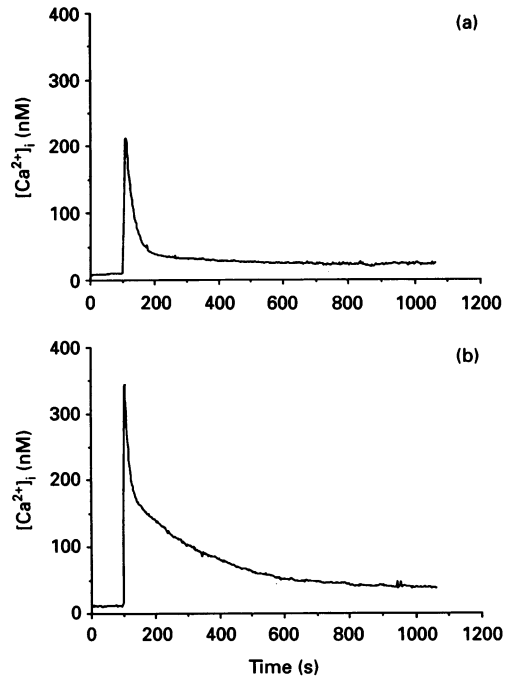

Figure 6 Regulation of $[Ca^{2+}]_i$ by long and short TRH-receptor isoforms

The regulation of $[Ca^{2+}]_i$ in single cells of clone 32 (long isoform) (**a**) and clone 1 (short isoform) (**b**) after application of TRH (1 μM) at $t = 100$ s was recorded as described in the Materials and methods section. Results from a typical experiment are displayed.

Table 1 Both the long and short isoforms of the rat TRH receptor are unable to regulate adenylate cyclase activity in membranes of receptor-expressing Rat 1 fibroblast clones

Adenylate cyclase activity was measured as described in the Materials and methods section in membranes of clones 1 and 32. Results are presented as means ± S.D. ($n = 4$). A similar lack of ability to regulate adenylate cyclase activity was observed in membranes from two further clones [clone 34, long isoform; clone 7, short isoform (results not shown)].

Condition	Adenylate cyclase activity (pmol/min per mg of membrane protein)	
	Long isoform (clone 32)	Short isoform (clone 1)
Basal	76 ± 0.1	100 ± 3.0
TRH (10 μM)	74 ± 0.8	99 ± 1.0
Forskolin (10 μM)	160 ± 3.0	220 ± 11.0
Forskolin (10 μM) + TRH (10 μM)	160 ± 5.0	230 ± 6.0

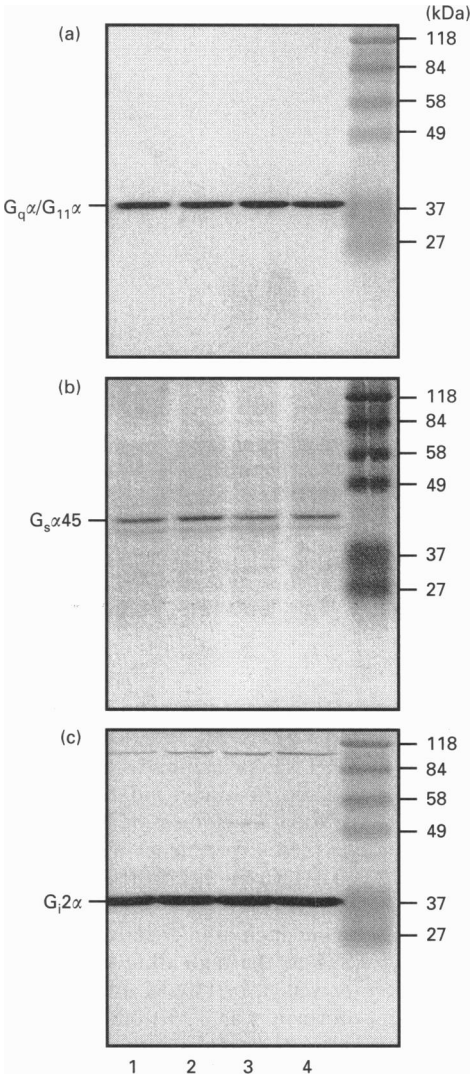

Figure 7 Rat 1 fibroblasts expressing either the long or the short isoforms of the Rat TRH receptor express similar levels of both phosphoinositidase C and adenylate cyclase regulatory G-proteins

Membranes (15 μg, panel **a**; 30 μg, panel **b**; or 20 μg, panel **c**) from Rat-1 fibroblast clone 32 (long isoform) (lanes 1 and 2) and clone 1 (short isoform) (lanes 3 and 4) were resolved by SDS/PAGE (10% acrylamide), transferred to nitrocellulose and subsequently immunoblotted with specific antisera to detect the presence of the α subunits of the phosphoinositidase C-linked G-proteins G_q/G_{11} (**a**), and the adenylate cyclase-stimulatory, G_s (**b**), and inhibitory, G_i2 (**c**), G-proteins.

The potential for either the long or short isoforms of the TRH receptor to regulate adenylate cyclase activity was also assessed in membranes of clones 1 and 32. TRH (10 μM) was unable, however, either to stimulate basal or to inhibit forskolin (10 μM)-amplified adenylate cyclase activity (Table 1). To examine if this lack of regulation of adenylate cyclase might reflect the G-protein profile of Rat 1 fibroblasts, we assessed the expression of the α subunits of the G-proteins associated with each of stimulation of phosphoinositidase C (G_q, G_{11}) and stimulation (G_s) and inhibition (G_i2, G_i3) of adenylate cyclase. Each of these polypeptides was demonstrated to be expressed in membranes of these cells (Figure 7, and results not shown).

TRH stimulation of the ERK-1 (MAP kinase) cascade has recently been reported in pituitary GH3 cells [25]. We confirmed this observation (Figure 8a), by demonstrating that addition of TRH (10 μM; 5 min) to GH3 cells resulted in the characteristic decrease in mobility of the ERK-1 (44 kDa MAP kinase) in SDS/PAGE gels which is routinely correlated with phosphorylation and activation of this protein [22–24]. In clones of Rat 1 cells expressing either the long or the short isoform of the TRH receptor which had been maintained in the absence of serum for 48 h, TRH (10 μM, 5 min) had only a small modulatory effect on the fraction of the cellular population of this polypeptide in the activated form (Figure 8b). Treatment of the cells with the phorbol ester, phorbol myristate acetate, equally resulted in only minimal modification. In parallel experiments both epidermal growth factor (10 nM) and lysophosphatidic acid (10 μM), which have both previously been noted to cause stimulation of ERK activity in Rat 1 cells and clones derived from them [24,26–28], produced this characteristic mobility shift in essentially the total cellular population of ERK-1 (Figure 8b). Pertussis-toxin (25 ng/ml, 16 h) treatment of the cells prevented lysophosphatidic acid regulation of ERK-1 mobility, but not that produced by epidermal growth factor (results not shown).

DISCUSSION

It has generally been observed that TRH acts as a phosphoinositidase-C-linked hormone, the receptor for which interacts with pertussis-toxin-insensitive G-proteins of the G_q class [4,5,29]. In a number of reports, however, particularly in GH3 and related rat anterior-pituitary cell lines, which are perhaps the most used model cell systems for analysis of TRH-receptor function, interactions of TRH receptors with other G-proteins [6,7], most prominently G_s and G_i2 (the G-proteins traditionally viewed as being responsible for stimulatory and inhibitory regulation of adenylate cylase) have been demonstrated.

The identification of expression of splice variants of the TRH receptor in GH3 cells [8] offered a potential explanation of these findings and begs the question of whether these splice variants display different functional properties. A number of molecularly distinct receptors are now appreciated to result from differential splicing of pre-mRNA derived from a single gene and, particularly in the cases of the splice variants of the EP3 prostanoid receptor [30] and the pituitary adenylate cyclase-activating-polypeptide (PACAP) receptor [31], very different second-mess-

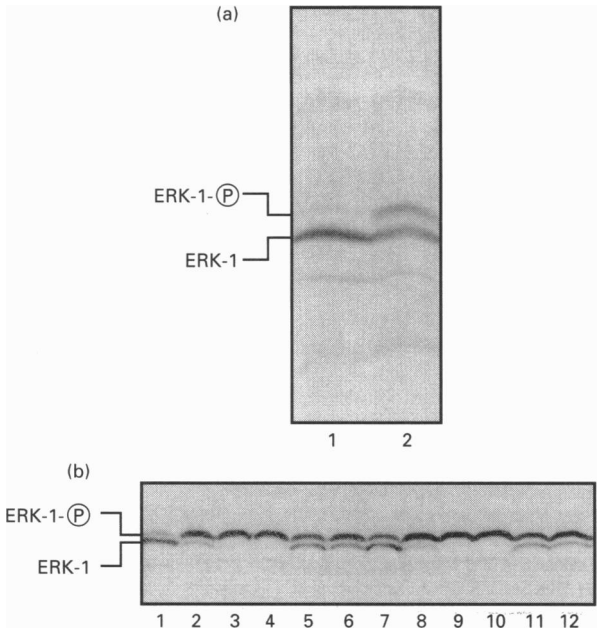

Figure 8 Neither the long or the short isoforms of the Rat TRH receptor is able to regulate ERK-1 substantially in Rat 1 fibroblasts

(**a**) TRH alters the gel mobility of ERK-1 in GH3 cells. GH3 cells were deprived of serum for 48 h and then challenged with (2) or without (1) TRH (10 μM) for 5 min. Cell lysates were resolved by SDS/PAGE (10% acrylamide containing 6M urea) and immunoblotted for the presence and mobility of ERK-1. After challenge with TRH a substantial fraction of this polypeptide migrated through the gel with decreased mobility, a characteristic associated with phosphorylation and activation of this polypeptide. (**b**) Epidermal growth factor (EGF) and lysophosphatidic acid, but not TRH or phorbol myristate acetate, regulate ERK-1 in Rat 1 fibroblasts expressing short or long forms of the TRH receptor. Cells of clone 32 (TRH-receptor long isoform) (lanes 1–6) and clone 1 (TRH-receptor short isoform) (lanes 7–12) were deprived of serum for 48 h and then subsequently challenged with vehicle (lanes 1, 7), lysophosphatidic acid (10 μM) (lanes 2, 8), EGF (10 nM) (lanes 3, 9), EGF (10 nM) plus TRH (10 μM) (lanes 4, 10), TRH (10 μM) (lanes 5, 11), or phorbol myristate acetate (1 μM) (lanes 6, 12) for 5 min. Cell lysates were resolved by SDS/PAGE (10% acrylamide containing 6M urea) and immunoblotted for the presence and mobility of ERK-1.

enger generation functions have been recorded for the individual forms. Splice variation in the rat TRH receptors results from the presence or absence of a 52-bp segment in the C-terminal tail to yield either a 412-amino-acid polypeptide (long isoform) or a 387-amino-acid version (short isoform) [8]. This is a reflection that the absence of a 52-bp fragment not only removes genetic information, but also results in a frame shift such that the amino acid sequence downstream of the delection is altered and a differed stop codon is encountered (which is not in frame in the long isoform).

In the present study we have examined whether the signalling characteristics of the two isoforms differ following stable expression of each form in Rat 1 fibroblasts, to assess whether reported regulation of effector systems other than phosphoinositidase C might reflect activation of one isoform rather than another. Our initial preconceptions were that this might be a property of the short isoform, as, assessed by RT-PCR, this is the minor form of the receptor in both normal pituitary and GH3 cells [8].

After isolation of clones expressing specific [^{3}H]TRH-binding sites, we initially determined that the clones expressed only the expected splice variant form of the receptor by reverse transcription of RNA isolated from these clones and subsequent PCR using a primer pair designed to generate fragments which would demonstrate the presence or absence of the 52-bp section which differs between the two isoforms [6,8] (Figure 2).

Both receptor isoforms displayed similar binding affinity for [^{3}H]TRH (Figure 3). Most of the binding experiments were performed in the presence of 100 μM p[NH]ppG. The rationale for this approach was that no radiolabelled antagonists are available for the TRH receptor, and when binding experiments with [^{3}H]TRH were performed in the absence of p[NH]ppG the data indicated a mixture of apparent low- and high-affinity sites with h values substantially below 1 (Figures 3c and 3d).

However, p[NH]ppG converts all G-protein-linked receptors into a G-protein-free state, which displays lower but uniform affinity for agonists and thus allows easier estimation of the binding parameters of $B_{max.}$ and K_d for a ligand. Under these conditions the h values for the self-competition experiments were close to 1. These were assessed by using the formalisms of DeBlasi et al. [32] as these relate specifically to self-competition experiments in which the h value from the data is close to 1.0 (see the legends to Figures 3a and 3b for confirmation of the suitability of this approach in this case). However, even under such conditions the estimated values for the binding parameters should be treated with a degree of caution, as agonist-binding characteristics are difficult to analyse unambiguously. Under these conditions both the short and long isoforms of the rat TRH receptor bound TRH with an estimated K_d of some 80 nM (Figure 3). We also performed a series of [^{3}H]TRH-binding experiments in the absence of p[NH]ppG and analysed these data using two-site displacement models. The estimated K_d for TRH at the high-affinity state for each isoform was close to 10 nM. These values are similar to the measured EC_{50} of 5–10 nM for TRH stimulation of inositol phosphate production observed for clones expressing both the long and short TRH-receptor splice variants (Figure 4), and hence indicate that it is the high-agonist-affinity state of the receptor which is able to interact with the cellular G-protein population to produce effector regulation. This similarity of EC_{50} values for TRH-mediated stimulation of inositol phosphate production is in agreement with analysis of TRH regulation of Cl^- flux in *Xenopus* oocytes after injection of cRNA species corresponding to the two isoforms [8]. Given the location of the splice variation site in the C-terminal tail and the demonstration that Tyr-106 in the third transmembrane helix of the receptor plays a key role in the binding of TRH [33], it should not be considered surprising that the two splice variants display very similar binding and activation affinities for TRH.

In contrast with the reported activation of adenylate cyclase by TRH in GH cells, which has been demonstrated to occur via a direct activation of $G_s\alpha$ [6], we were unable to record any ability of either TRH-receptor splice variant to alter adenylate cyclase activity (either positively or negatively) in membranes of the TRH receptor expressing clones of Rat-1 cells (Table 1). This is not a reflection of a lack of expression of the relevant G-proteins, which we have demonstrated to be expressed by these cells (Figure 7). It may of course be a reflection of the adenylate cyclase isoform distribution in the individual cell lines, an area which remains essentially uncharted, but, as all forms of hormonally regulated adenylate cyclase are activated by G_s [34], this seems unlikely. We must therefore conclude that neither isoform of the rat TRH receptor displays appreciable ability to interact with either G_s (or indeed with G_i2) at least when expressed in this genetic background. As such we can provide no evidence in this study for either of the splice variants of the TRH receptor providing a locus for bifurcation of signalling information, as has recently been described for a variety of receptors, including the TSH receptor [35] (see [36] for review).

Recent data have also noted the ability of TRH to stimulate MAP kinase (ERK) activity in GH3 cells [25], but it was unable to produce a significant effect in clones of Rat 1 cells expressing either the long or short isoforms of the rat TRH receptor (Figure 8). In such cells it has previously been reported that both receptors with intrinsic tyrosine kinase activity (the epidermal growth factor receptor) and G-protein-coupled receptors, which are able to cause activation of $G_i2\alpha$ and hence inhibition of adenylate cyclase activity (e.g. the endogenously expressed receptor for lysophosphatidic acid or, following stable transfection, the α_2C10 adrenoceptor), cause activation of this cascade [26–28]. It has also previously been noted that the endogenously expressed receptor for the peptide endothelin, and indeed stimulation of the cells with phorbol esters, has little ability to cause activation of ERK in these cells [24]. Thus, as TRH also functions to cause activation of a phosphoinositidase C, and thus stimulation of protein kinase C, it is not surprising that in this genetic background neither of the TRH-receptor isoforms regulated a signalling cascade necessary to produce this effect.

The data produced herein demonstrate that no obvious functional differences are observed for the long and the short isoforms of the rat TRH receptor, at least when expressed in Rat-1 fibroblasts, and further demonstrate a high selectivity of coupling of both of these isoforms to G_q/G_{11} stimulation of phosphoinositidase C over regulation of adenylate cyclase.

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