The effects of pyroglutamylglutamylprolineamide, a peptide related to thyrotrophin-releasing hormone, on rat anterior pituitary cells in culture

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Abstract

Pyroglutamylglutamylprolineamide, which was first discovered in mammalian prostate, differs from thyrotrophin-releasing hormone (TRH) by substitution of glutamic acid for histidine at position two of the tripeptide. Recently, the newly discovered peptide has been identified in substantial concentrations in the rat anterior pituitary gland and, in this study, we have investigated the effects of the peptide on rat anterior pituitary cells in culture. GH_3 cells were chosen to examine the possible effects of the new peptide, particularly in relation to its effects on the TRH receptor. This cell-type was deficient, in comparison with normal rat pituitary cells, in the new TRH-related peptide and appeared to be an ideal model cell in which to study the effects of pGlu-Glu-$ProNH_2$. TRH (0·01–100 nM) was found to stimulate the secretion of both GH and prolactin from GH_3 cells whereas pGlu-Glu-$ProNH_2$ had no effect within the same concentration ranges. In contrast, at micromolar concentrations pGlu-Glu-$ProNH_2$ exhibited intrinsic TRH-like activity causing stimulation of both GH and prolactin release from GH_3 cells. Both TRH and pGlu-Glu-$ProNH_2$ appeared to act through the same intracellular signalling mechanism, causing significant increases in intracellular inositol phosphate within the expected concentration ranges. However, pGlu-Glu-$ProNH_2$ (up to 1 mM) displaced neither [^{3}H]TRH nor [^{3}H]MeTRH from membrane-binding sites on GH_3 cells, suggesting that the effects of the new peptide were mediated through a second receptor. The physiological relevance of these effects of pGlu-Glu-$ProNH_2$ requires further investigation.

Journal of Endocrinology (1994) **142,** 111–118

Introduction

Thyrotrophin-releasing hormone (TRH) has potent biological activity both within the hypothalamo-pituitary axis and the central nervous system (CNS). A number of laboratories have succeeded in raising antibodies to TRH (pGlu-His-$ProNH_2$) by coupling through the imidazole ring of histidine (Bassiri & Utiger 1972) and these antibodies generally display specificities for the terminal amino acids but are tolerant of the residue at position 2. During the last decade, a series of reports have indicated that TRH antibodies are capable of detecting peptides with different chromatographic properties from authentic TRH. The first of these peptides, pGlu-Glu-$ProNH_2$, was purified from rabbit prostate and differs from TRH by the substitution of glutamic acid for histidine at position 2 (Cockle *et al.* 1989*a*). The novel peptide occurs in high concentrations in mammalian prostate and semen and its induction at sexual maturity suggests an important role in male fertility (Cockle *et al.* 1989*a*,*c*, Cockle *et al.* 1989*b*, Thetford *et al.* 1992). In this context, recent evidence strongly suggests that the new peptide is biologically active, enhancing the fertilizing ability of spermatozoa; furthermore, results indicate the presence of a specific receptor for the new peptide which is distinct from that for TRH (Green *et al.* 1994).

In view of the diverse distribution of TRH throughout the CNS and peripheral tissues (Hokfelt *et al.* 1989), we have recently examined a number of tissues for the presence of pGlu-Glu-$ProNH_2$. During the course of this study we have identified substantial concentrations of a peptide with identical chromatographic and immunological properties to pGlu-Glu-$ProNH_2$ in the anterior lobe of the rat pituitary gland: the peptide has been purified and its composition confirmed by amino acid analysis (Ashworth *et al.* 1991*a*). The new peptide is the major TRH immunoreactive peptide in the anterior pituitary gland whereas only trace amounts could be detected in rat hypothalamus (Ashworth *et al.* 1991*a*). Thus, it is likely that pGlu-Glu-$ProNH_2$ is derived from the pituitary gland and may be added to the list of bioactive peptides which modulate pituitary function by paracrine or autocrine mechanisms (Jones *et al.* 1990). During the present study, we investigated the effects of

  Printed in Great Britain
0022–0795/94/0142–0111 $08.00/0

pGlu-Glu-ProNH$_2$ on the secretion of anterior pituitary hormones and the mechanism of action of these effects. A portion of this work has been presented as a preliminary abstract (Ashworth *et al.* 1993).

Materials and Methods

Materials

GH_3 cells were obtained from Dr J Ham (University College of Wales, Cardiff, UK). Tissue culture plates, media and sera were obtained from Gibco BRL (Paisley, Strathclyde, UK) and radioimmunoassay (RIA) reagents for growth hormone (GH), prolactin and thyrotrophin (TSH) from NIDDK (Baltimore, MA, USA). TRH antibodies were kind gifts from Dr H Fraser (MRC Reproductive Biology, Edinburgh, UK) and Professor T J Visser (Erasmus University, Rotterdam, The Netherlands). Synthetic TRH was purchased from Cambridge Research Biochemicals (Northwich, Cheshire, UK) and pGlu-Glu-ProNH$_2$, poly-L-lysine, protein A, cyclic AMP (cAMP) RIA reagents and RIA grade bovine serum albumin (BSA) from Sigma (Poole, Dorset, UK). Sheep anti-rabbit IgG Fc antibody was provided by ImmunoGen International Ltd (Llandysul, Dyfed, UK). Sephadex resins were obtained from Pharmacia-LKB (Milton Keynes, Bucks, UK), TRH (L-prolyl-2,3,4,5-^{3}H(N)), referred to as [^{3}H]TRH, and 3-methyl histidyl-TRH (L-histidyl-4-^{3}H(N),L-prolyl-3,4-^{3}H(N)), referred to as [^{3}H]MeTRH, from NEN-Dupont (Stevenage, Herts, UK) and myo-[^{3}H] inositol from Amersham International plc (Amersham, Bucks, UK).

Analysis of GH_3 cells for pGlu-Glu-ProNH$_2$

GH_3 cells were seeded into 40 flasks (75 cm^2) and grown as described below until cells approached confluency. The approximate wet weight of cells used for the analysis was 1·0 g. The cells were washed with phosphate-buffered saline (PBS) and then 10 ml acidified acetone (H_2O:HCl:acetone Analar; 5:1:40) was added to each flask and the cells removed by scraping. The extract was centrifuged at 4 °C, 30 000 *g* for 30 min and the supernatant stored at −20 °C for less than 4 days before analysis. TRH and pGlu-Glu-ProNH$_2$ in the cell extract were separated by anion-exchange chromatography (Ashworth *et al.* 1991*a*). Aliquots of each fraction were dried *in vacuo* and pGlu-Glu-ProNH$_2$ detected by RIA as described previously (Ashworth *et al.* 1991*b*).

Cell culture

GH_3 cells were maintained and subcultured as described previously (Tashjian *et al.* 1970) in Ham's F10 growth medium, buffered with sodium bicarbonate and containing 15% horse serum, 2·5% fetal calf serum (FCS) and penicillin (100 IU/ml) and streptomycin (100 mg/ml). Cells, both GH_3 and normal anterior pituitary cells (see below), were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. Six days prior to the experiment, GH_3 cells were seeded at a density of 5×10^4 cells/well in 24-well plates (precoated with poly-L-lysine) containing 0·5 ml growth medium/well: the cells were allowed to grow with one change of medium on day 3. On the day of the experiment, the medium was replaced with 0·5 ml serum-free Ham's F10 containing 0·25% BSA (RIA grade), 25 mM Hepes and antibiotics (experimental medium). After a 2-h equilibration period, the experimental medium was replaced with 0·5 ml of the same medium with or without peptide hormone additives. At the end of a 2 h incubation the medium was removed from the cells, centrifuged at 100 *g* and aliquots of the supernatant were stored at −20 °C until RIA of anterior pituitary hormones.

Primary cultures of normal anterior pituitary cells from female rats (Sprague–Dawley) were established according to the method of Kirkland & Ellison (1981). The cells, in 0·5 ml Dulbecco's Modified Eagles' Medium (DMEM) buffered with sodium bicarbonate and containing 10% FCS, kanamycin (100 μg/ml) and fungizone (2·5 μg/ml), were plated at a density of 3×10^5/well in 24-well plates (uncoated) and allowed to stabilize for 72 h before the experiment. The experiment was performed as described above except that the experimental medium was DMEM containing 25 mM Hepes, 0·25% BSA (RIA grade), fungizone and kanamycin.

RIA of anterior pituitary hormones

Rat GH, prolactin and TSH, standards, antisera and hormones for radioiodination were obtained from NIDDK. Radioiodination and assays were performed as described in the instructions provided with each kit. The data were processed on an Elonex PC-320X using Riacalc software (Pharmacia/LKB Wallac, Turku, Finland). The precision of the assays was assessed by measuring both inter- (<14% for all assays) and intra-assay (<9% for all assays) coefficients of variation.

Stability of pGlu-Glu-ProNH$_2$ in tissue culture medium

GH_3 cells, grown and seeded into 24-well plates as described above, were incubated with experimental medium containing 250 μM pGlu-Glu-ProNH$_2$ for 2 h. The concentrations of the peptide before and after incubation was assessed by the absorbance (LKB variable wavelength detector) of the peptide peak at 215 nm (and confirmed by RIA of fractions as described previously: Ashworth *et al.* 1991*b*) after high-performance liquid chromatography (HPLC). In brief, the peptide in each

sample was resolved by reverse phase HPLC on a Hypersil C18 reverse phase column (25 × 0·46 cm; Highchrom Ltd, Reading, Berks, UK) using a linear gradient (0–20%, 1% per min) of water–acetonitrile containing 0·1% trifluoroacetic acid. The flow rate was 1 ml/min and fractions were collected every 30 s.

Analysis of total cellular inositol monophosphate and cAMP

GH_3 cells were grown and seeded into 24-well plates as described above. Cells were exposed to 10 μCi myo-[^{3}H]inositol/plate in the same culture media for 7 days. Prior to experimentation, the supernatant containing the radioactivity was removed and the cells washed in Earle's Balanced Salt Solution (EBSS). The cultures were exposed to the peptides in EBSS for 30 min at 37 °C in the presence of 10 mM LiCl and then radioactive inositol monophosphate was measured by the method of Berridge *et al.* (1983). cAMP levels in cells treated with TRH (up to 10^{-6} M) and pGlu-Glu-ProNH$_2$ (up to 10^{-3} M) for up to 30 min were measured by specific RIA using an antibody raised against succinyl cAMP conjugated to BSA. The IgG antibody fraction was prepared by precipitation of the serum with ammonium sulphate (Rees-Smith & Hall 1981). Assay samples were treated with triethylamine and acetic anhydride prior to assay (Brooker *et al.* 1979). The antibody was used at a final dilution of 1:50 000 and the bound cAMP was precipitated with polyethylene glycol (PEG; mol.wt 6000). Sensitivity of the assay was <4 fmol/tube.

Analysis of [^{3}H]TRH binding

GH_3 cells were grown and seeded into 24-well plates as described above. On the day of the experiment, the cells were preincubated for 15 min with EBSS (0·5 ml/well) containing 0·4% BSA (RIA grade). The medium in each well was then replaced with 0·25 ml EBSS containing 0·4% BSA (RIA grade) and [^{3}H]TRH (83 Ci/mmol) or [^{3}H]MeTRH (87 Ci/mmol), with or without peptide additions at a range of concentrations. A number of experiments was performed in which the cells were incubated for 30 min with concentrations of radiolabelled ligands in the range 1–10 nM. The cells were then washed twice in 1·0 ml PBS at 0 °C and then solubilized in 0·5 ml 10% sodium dodecyl sulphate and radioactivity in each sample was determined by liquid scintillation counting.

Statistical analyses

All results are expressed as means ± S.E.M. Statistical significance was assessed by analysis of variance (ANOVA) and paired Student's *t*-test where appropriate. Analyses were performed using Statworks software designed for the Apple Macintosh and the differences were judged significant when P was 5% or less.

Results

Analysis of GH_3 cells

We were interested in the possibility that the newly discovered peptide may act as a paracrine or autocrine modulator at the level of the pituitary; in particular, in the idea that it could modulate the actions of TRH perhaps through the TRH receptor. The GH_3 cell line which is derived from a rat anterior pituitary tumour was chosen as the model system because it is known to contain high densities of functional TRH receptors (Hinkle & Tashjian 1975). Recently, substantial levels of pGlu-Glu-ProNH$_2$ have been detected in the normal rat anterior pituitary gland (Ashworth *et al.* 1991*a*), and for this reason it was appropriate to determine the level of the peptide in GH_3 cells. TRH-related peptides in cell extracts were separated by anion-exchange chromatography under conditions which allow complete resolution of TRH and pGlu-Glu-ProNH$_2$. Aliquots of each fraction were then assayed for the peptide using an antibody which displays a higher specificity for pGlu-Glu-ProNH$_2$ than authentic TRH (Ashworth *et al.* 1991*b*). In this way, GH_3 cells were found to contain only low levels of pGlu-Glu-ProNH$_2$ (0·19 pmol/g) compared with normal anterior pituitary cells which are known to contain substantial levels of the peptide (5·8 pmol/g; Ashworth *et al.* 1991*a*). Authentic TRH and neutral TRH-related peptides were not detected.

Effect of nanomolar concentrations of pGlu-Glu-ProNH$_2$

Because of the structural similarities of the two peptides, it was important to determine whether pGlu-Glu-ProNH$_2$ displayed intrinsic TRH-like activity. The peptides (up to 100 nM) were incubated for 2 h with GH_3 cells. As expected, TRH induced significant release of both GH and prolactin in a dose-dependent manner with optimal effects observed between 10 and 100 nM. However, pGlu-Glu-ProNH$_2$ at concentrations up to 100 nM had no significant effect on secretion of either hormone from GH_3 cells (Fig. 1). Similar experiments were repeated in primary cultures of normal anterior pituitary cells: TRH stimulated secretion of TSH and prolactin from normal cells in a dose-dependent manner, reaching optimal release at concentrations of peptide between 10 and 100 nM. As in the case of GH_3 cells, pGlu-Glu-ProNH$_2$ had no intrinsic TRH-like activity in cultures of normal cells (Fig. 2).

Effect of micromolar concentrations of pGlu-Glu-ProNH$_2$

The novel peptide may not be acting in a classical endocrine manner but may have an autocrine or paracrine function. In this situation, the peptide would be expected to be secreted into a discrete microenvironment where it may be present in a high local concentration. Thus, it was

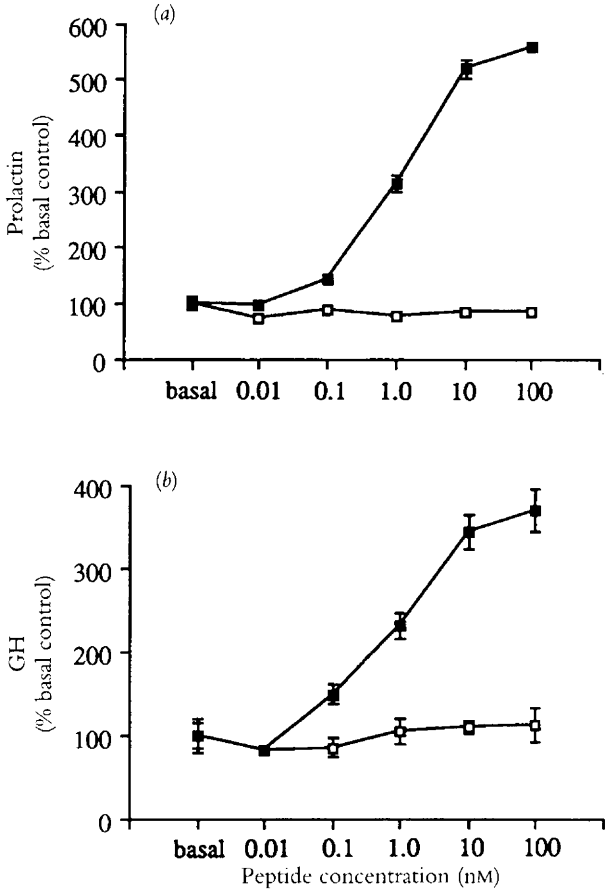

FIGURE 1. The effect of TRH (■) and pGlu-Glu-ProNH$_2$ (□), 0–100 nM, on (*a*) prolactin and (*b*) GH release from GH$_3$ cells. The peptides were incubated with cells for 2 h, after which the medium was removed, centrifuged and the supernatant frozen until radioimmunoassay. TRH produced a significant dose-dependent release of both hormones ($P<0{\cdot}001$, ANOVA) whereas pGlu-Glu-ProNH$_2$ showed no significant increase. Data are presented as means ± S.E.M. where $n=4$.

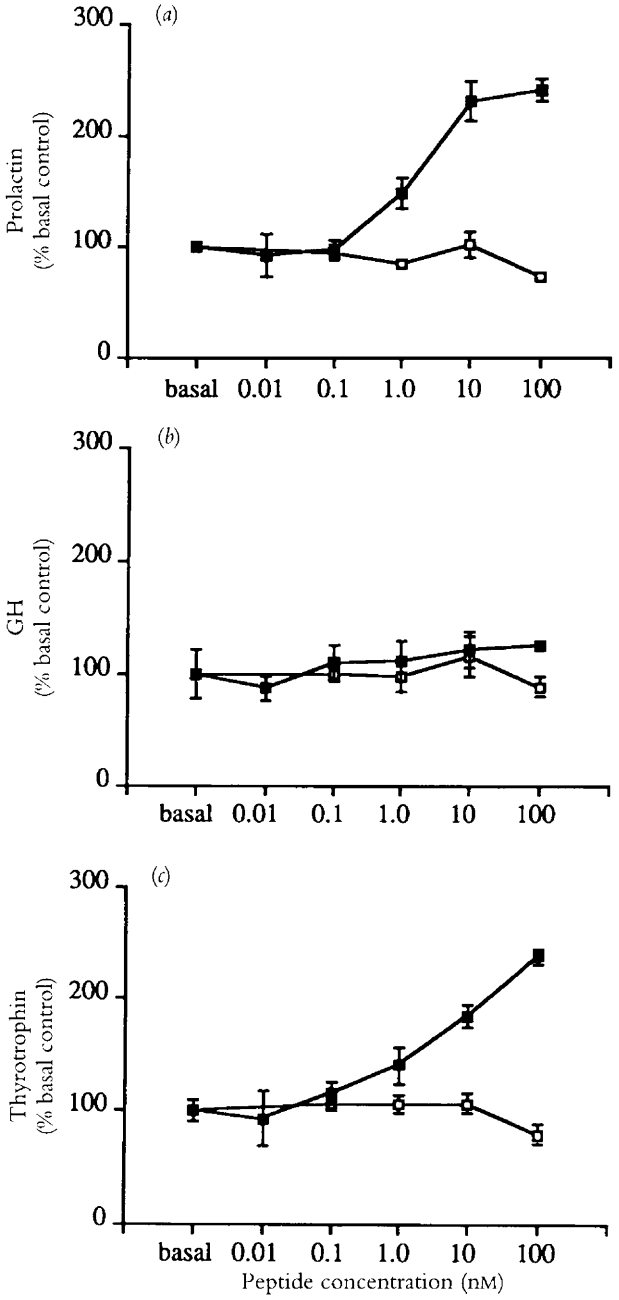

FIGURE 2. The effect of TRH (■) and pGlu-Glu-ProNH$_2$ (□), 0–100 nM, on (*a*) prolactin, (*b*) GH and (*c*) TSH release from rat anterior pituitary cells grown in culture. The pituitary cells were prepared from the glands from ten female Sprague–Dawley rats. The peptides were incubated with the cells for 2 h after which the medium was removed, centrifuged and the supernatant frozen until radioimmunoassay. TRH produced a significant increase in the release of prolactin and TSH ($P<0{\cdot}001$, ANOVA) but had no effect on GH (basal secretion 161·0 ± 8·2 ng/ml). pGlu-Glu-ProNH$_2$ had no effect on secretion of any of the pituitary hormones. Data are presented as means ± S.E.M. where $n=4$.

interesting to explore possible activity of micromolar concentrations of pGlu-Glu-ProNH$_2$. As illustrated in Fig. 3, concentrations of pGlu-Glu-ProNH$_2$ of above 100 μM induced secretion of both GH and prolactin from GH$_3$ cells. Maximal response was observed between 1 and 4 mM. In contrast, the novel peptide at these concentrations had no effect on static cultures of normal anterior pituitary cells.

Both peptides are degraded rapidly by proteases present in biological fluids, although generally pGlu-Glu-ProNH$_2$ is more stable than TRH (Ashworth *et al.* 1991*b*). A primary metabolite of TRH is His-Pro diketopiperazine which has biological activity (for review see Prasad 1988), and similarly, Glu-Pro diketopiperazine is likely to be a major peptide formed from pGlu-Glu-ProNH$_2$. The effects described above may thus be due to a degradation

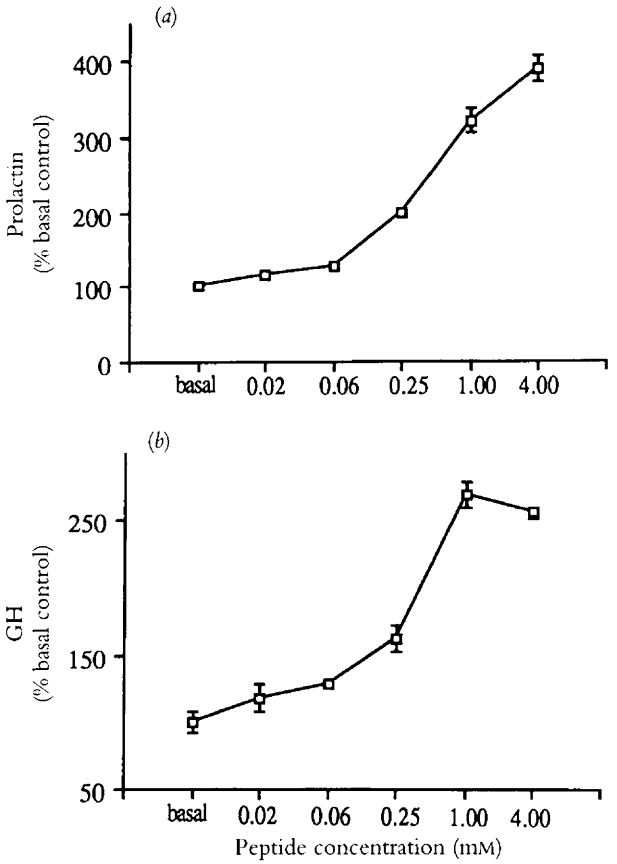

FIGURE 3. The effect of high doses of pGlu-Glu-ProNH$_2$ on (*a*) prolactin and (*b*) GH secretion from GH$_3$ cells. The peptides were incubated with the cells for 2 h after which the medium was removed, centrifuged and frozen until radioimmunoassay. pGlu-Glu-ProNH$_2$ produced a significant increase in the release of both hormones ($P<0.001$, ANOVA). Data are presented as means ± S.E.M. where $n=4$.

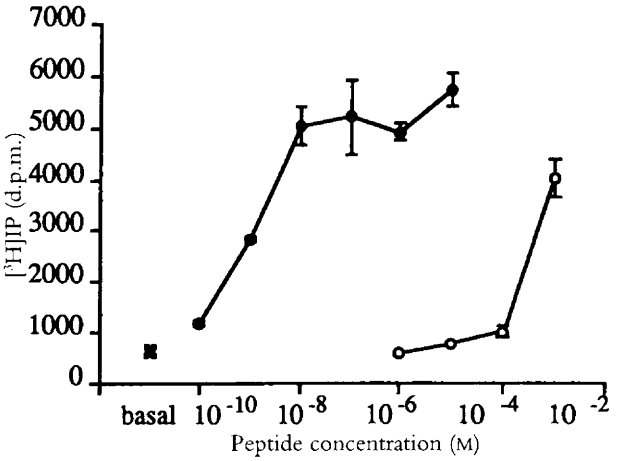

FIGURE 4. The effect of TRH (●, 0·1 nM–10 μM) and pGlu-Glu-ProNH$_2$ (○, 10 μM–1 mM) on inositol phosphate (IP) formation in GH$_3$ cells. Both peptides caused a significant increase in the formation of inositol phosphate after incubation with the cells for 30 min ($P<0.001$, ANOVA). Data are presented as means ± S.E.M. where $n=4$.

product rather than the tripeptide itself. In order to investigate this possibility, the degradation of pGlu-Glu-ProNH$_2$ was assessed after incubation with GH$_3$ cells, as described in Materials and Methods. Degradation of pGlu-Glu-ProNH$_2$ was not observed during the experimental period; furthermore, TRH is known to remain intact over the same period displaying only 1% degradation in 3 days (Dannies & Tashjian 1976).

Effect of pGlu-Glu-ProNH$_2$ on cellular inositol phosphate and cAMP

It is now generally accepted that the effects of TRH are mediated through the phosphoinositol pathway (Gershengorn 1986) although cAMP may be involved in the GH response to TRH in domestic fowl (Perez *et al.* 1989). In order to dissect the mechanism of action of pGlu-Glu-ProNH$_2$, we have investigated the second messenger response in terms of production of inositol phosphate and cAMP. Both TRH and pGlu-Glu-ProNH$_2$ stimulated the formation of inositol phosphate in GH$_3$ cells as illustrated in Fig. 4. The two peptides were effective in the expected concentration ranges: TRH at concentrations above 0·1 nM induced significant increases in inositol phosphate reaching maximal levels at 10–100 nM peptide. In contrast, pGlu-Glu-ProNH$_2$ caused significant increases in inositol phosphate only at concentrations above 100 μM. Changes in cellular cAMP were not observed in GH$_3$ cells after treatment with either peptide.

Binding of [^{3}H]TRH and [^{3}H]MeTRH to GH$_3$ cells

Results outlined above indicate that the novel peptide may operate through a similar signalling mechanism to that of TRH, and thus the question arose as to whether the two peptides also acted through the same receptor. For this reason, the ability of pGlu-Glu-ProNH$_2$ to compete with both high ([^{3}H]MeTRH) and low affinity ([^{3}H]TRH) receptor ligands was assessed using GH$_3$ cells. As illustrated in Fig. 5, pGlu-Glu-ProNH$_2$ was unable to compete with [^{3}H]TRH (10 nM) for TRH-binding sites whereas synthetic TRH at concentrations above 10 nM could significantly displace label. Similar results were observed using 2·5 nM radiolabelled ligand; for example, pGlu-Glu-ProNH$_2$ (1 mM) was unable to displace radioactivity from GH$_3$ cells, even though TRH could compete more

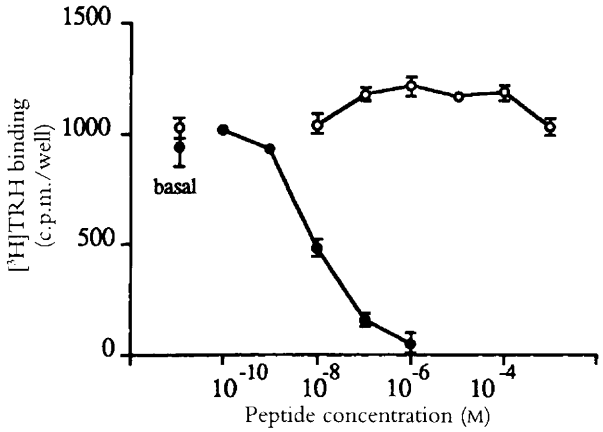

FIGURE 5. The effect of TRH (●, 0·1 nM–1 μM) and pGlu-Glu-ProNH$_2$ (○, 10 nM–1 mM) on the binding of [^{3}H]TRH to GH$_3$ cells. The cells were incubated with the label and peptides for 30 min and then the cells were washed with phosphate-buffered saline at 0 °C before estimation of bound radioactivity. TRH (1 μM) caused the complete displacement of label from the cells whereas pGlu-Glu-ProNH$_2$ had no significant effect even at a concentration of 1 mM. Data are presented as means ± S.E.M. where n=4.

effectively with the lower concentration of [^{3}H]TRH. The results were essentially the same for [^{3}H]MeTRH, although this peptide has a higher affinity for the TRH receptor and, as expected, higher concentrations of synthetic TRH were required to displace the bound radioactivity. Even at high concentrations (1 mM), pGlu-Glu-ProNH$_2$ was unable to compete with the methylated ligand (1 and 6·7 nM).

Discussion

During an on-going study to investigate the distribution of TRH-related peptides, pGlu-Glu-ProNH$_2$ was recently identified in the rat anterior pituitary gland where it represents 63% of total TRH immunoreactivity; in contrast, the peptide is a minor component of TRH immunoreactivity in the posterior lobe and present in only trace amounts in the hypothalamus (Ashworth *et al.* 1991*a*). Recently, it has been confirmed that pGlu-Glu-ProNH$_2$ is the major TRH-immunoreactive peptide in the rat anterior pituitary gland (Rondeel *et al.* 1993). Furthermore, the peptide has been identified in the pituitary glands of other species (Ashworth *et al.* 1991*a*,*b*, Harvey *et al.* 1993) and we speculate that pGlu-Glu-ProNH$_2$ released locally may play a general role in modulating the activity of this gland. In support of this hypothesis, pituitary pGlu-Glu-ProNH$_2$ has been shown to vary in a number of pathological conditions (Rondeel *et al.* 1993).

In the current study, we have examined the effects of pGlu-Glu-ProNH$_2$ on secretion of hormones from both GH$_3$ and normal anterior pituitary cells. Although nanomolar concentrations of pGlu-Glu-ProNH$_2$ displayed no intrinsic TRH-like activity, at concentrations above 100 μM, the peptide caused a significant increase in the secretion of both GH and prolactin from GH$_3$ cells. This activity is likely to be due to the intact peptide, because degradation of pGlu-Glu-ProNH$_2$ was not observed during the incubation period.

Stimulation of inositol phosphate production, but not of cAMP, was also observed after incubation of GH$_3$ cells with concentrations of the peptide that induce secretion; thus, the effects of the peptide appear to be mediated through the same signalling mechanism as TRH itself. It has been suggested that the chemical nature of all three amino acid residues are essential for binding to the TRH receptor (Hinkle *et al.* 1974), and indeed the integrity of the histidine moiety would appear to be crucial because radioiodinated TRH exerts none of the physiological effects of native TRH (Gourdi *et al.* 1973). In agreement with the above studies, we found that, even at high concentrations, the novel peptide was unable to compete with either [^{3}H]TRH or [^{3}H]MeTRH for TRH-binding sites on GH$_3$ cells and is thus unlikely to act through the same receptor as TRH.

Surprisingly, high concentrations of pGlu-Glu-ProNH$_2$ have recently been shown to be capable of competing with [^{3}H]MeTRH for the TRH receptor in the membranes of anterior pituitary cells from domestic fowl (Harvey *et al.* 1993). The difference between the rat and avian receptor binding studies are difficult to explain at present because, to date, pituitary receptors have been cloned and sequenced only for rodents (Straub *et al.* 1990, de la Pena *et al.* 1992, Zhao *et al.* 1992, Sellar *et al.* 1993) and man (Duthie *et al.* 1993, Matre *et al.* 1993). From these studies, it appears that TRH receptors reveal a high degree of homology, but with differences at the carboxy terminus; whether changes in this region can modify the binding of TRH-related peptides remains to be determined.

Although pGlu-Glu-ProNH$_2$ demonstrated clear intrinsic TRH-like activity in terms of GH and prolactin release from GH$_3$ cells, the concentrations required for the effect were higher than might be expected for a physiological response; for example, optimal effects on cells in culture are generally observed within the nanomolar range for TRH (Martin & Tashjian 1977). However, a direct comparison between the effective concentrations for TRH and pGlu-Glu-ProNH$_2$ may be inappropriate if the peptide acts in an autocrine or paracrine manner; for example, pGlu-Glu-ProNH$_2$ may be released in very high concentrations into the restricted extracellular space of the pituitary gland. In this context, it is interesting that a response was observed in GH$_3$ cells which are deficient in the peptide but not in normal pituitary cells which

contain substantial levels of pGlu-Glu-ProNH$_2$. Such findings may be consistent with down-regulation of the appropriate receptor in cells which secrete high levels of the peptide. In the future, the availability of [^{3}H]pGlu-Glu-ProNH$_2$ for analysis of the binding site may help to clarify the relevance of the intrinsic TRH-like activity of pGlu-Glu-ProNH$_2$. In summary, pGlu-Glu-ProNH$_2$ displayed weakly agonistic activity in GH$_3$ cells, inducing secretion of both GH and prolactin with concomitant increases in intracellular inositol phosphate. Furthermore, these actions were not mediated through the TRH receptor and it is likely that a second receptor was involved.

Acknowledgements

We thank Dr H Fraser and Professor T J Visser for their kind gifts of TRH antisera, NIDDK for their RIA kits, and The Wellcome Trust (036602) and Medical Research Council (G8719998SB) for supporting this work.

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Revised manuscript received 20 December 1993
Accepted 2 February 1994