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The thyrotropin-releasing hormone-like peptides pGlu-Phe-Pro amide and pGlu-Glu-Pro amide increase plasma triiodothyronine levels in the mouse; the activity is sensitive to testosterone

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Abstract

Three naturally occurring peptides, pGlu-Glu-Pro amide, pGlu-Phe-Pro amide and pGlu-Gln-Pro amide, with similar structures to thyrotropin releasing hormone (TRH) have recently been identified but no studies of their in vivo activities have been reported previously. We describe here the ability of pGlu-Phe-Pro amide and pGlu-Glu-Pro amide to influence thyroid status. Subcutaneous administration of these 'TRH-like' peptides in male and female CD1 mice led to increased levels of triiodothyronine (T3) and to a lesser extent tetraiodothyronine (T4) in the circulation. pGlu-Phe-Pro amide was more potent than pGlu-Glu-Pro amide; it exhibited a similar potency to pGlu-His-Pro amide (TRH). pGlu-Phe-Pro amide, pGlu-Glu-Pro amide and TRH produced significantly greater effects in the female than in the male. Castration of male mice led to increased activities, with potencies comparable to those seen in the female; in contrast treatment of female mice with testosterone resulted in reduced activities, similar to those observed in the control male. The effects of potassium deprivation on the activities of the TRH-like peptides were also investigated. This diet, which results in decreased testosterone levels in the male, led to increased activities of the TRH-like peptides and TRH, approaching the potencies observed in the female. The results demonstrate that the TRH-like peptides pGlu-Phe-Pro amide and pGlu-Glu-Pro amide which occur naturally in the thyroid gland exhibit biological activity in influencing thyroid status in vivo. The activities are sensitive to testosterone.

Keywords: TRH-like peptide; TRH (thyrotropin releasing hormone); Thyroid status; Testosterone; K^+ deprivation

1. Introduction

Recent studies have led to the identification of three pyroglutamyl peptide amides with similar structures to thyrotropin-releasing hormone (TRH), two of which, pGlu-Phe-Pro amide and pGlu-Glu-Pro amide, were identified unequivocally by mass spectrometry. In these peptides the central histidine residue of TRH is replaced by glutamic acid (Cockle et al., 1989), glutamine or phenylalanine (Khan et al., 1992) and they retain much or all of the immunoreactivity of TRH. These 'TRH-like' peptides occur principally in the male reproductive system but are also present in a variety of other tissues (Del Rio-Garcia and Smyth, 1990). Our understanding of their physiological roles, however, is not yet complete. It has been reported that pGlu-Glu-Pro amide can increase the capacitation of mouse (Green et al., 1994) and human (Green et al., 1996) sperm cells in vitro, consistent with its presence in seminal fluid and a possible role in fertility. More recently it has been shown that the levels of pGlu-Glu-Pro amide, present in relatively low concentration in rat anterior pituitary, are sensitive to the sexual hormones, testosterone and oestradiol (Akinsanya et al., 1995a; Rondeel et al., 1995) and to dexamethasone (Akinsanya et al., 1995b), indicating that this peptide is regulated by different hormonal systems. In addition, the release of growth hormone from GH3 pituitary cells, which is known to be stimulated

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by TRH, is also stimulated by pGlu-Glu-Pro amide (Ashworth et al., 1994), albeit with lower potency.

We have shown that both TRH-like peptides and TRH are present in rat thyroid (Del Rio-Garcia and Smyth, 1990; Rausell et al., 1998). The finding of these peptides in a target tissue at the end of the hypothalamic–hypophyseal thyroid axis was unexpected and raised the possibility that thyroidal TRH or TRH-like peptides might be involved in the regulation of thyroid status. In this communication we describe the effects of pGlu-Glu-Pro amide and pGlu-Phe-Pro amide, administered subcutaneously, in raising the plasma levels of triiodothyronine (T3) and tetraiodothyronine (T4) in the mouse and we compare their activities with that of TRH. In addition, we report differences in the responses produced in the male vs. the female mouse and we describe the effects of castration, testosterone administration and potassium deficiency on the activities of these peptides.

2. Materials and methods

The TRH-like peptides pGlu-Phe-Pro amide and pGlu-Glu-Pro amide, were synthesized in the Laboratory of Peptide Chemistry at the National Institute for Medical Research, Mill Hill, London, UK. The solid phase method employed involved Fmoc protection of NH_2-groups and *t*-butyl ester protection of the carboxyl group of glutamic acid, and coupling was effected by dicyclohexylcarbodiimide (DCC). After release from the resin support by trifluoracetic acid, the peptides were purified by high performance liquid chromatography (HPLC) and their purity confirmed by amino acid analysis and mass spectrometry.

CD1 mice were used in this experimental study. Adult animals (60–80 days, 30–35 g) were given free access to standard chow and water and were exposed to a 12:12 h light:dark cycle under controlled conditions of temperature (22°C) and humidity (50%). In some experiments female mice were treated with testosterone propionate (100 mg/kg, p.d., Sigma) over a 14-day period by subcutaneous injection of the hormone in a solution of olive oil while control animals received vehicle alone. Potassium deficiency was produced by feeding mice on a diet similar to the controls but containing 120 mg of potassium/kg of food (UAR 212 K) while control animals were fed with standard chow (UAR A03) containing 7.5 g potassium/kg. Castration of male mice was performed under ether anaesthesia and experiments were carried out with a minimum period of 7 days after the operation.

To determine plasma levels of T3 and T4, blood was obtained by cardiac puncture under light ether anaesthesia. The levels were determined in control male and female mice and after treatment with pGlu-His-Pro amide (TRH, Sigma), pGlu-Phe-Pro amide or pGlu-Glu-Pro amide (100 μg in 300 μl of aqueous solution). Control animals re-

Table 1
T3 and T4 levels in CD1 adult female and male mice administered with pGlu-Phe-Pro amide, pGlu-Glu-Pro amide or pGlu-His-Pro amide (TRH)

	Pretreatment	Peptide	T3 (nmol/l)	*n*	T4 (nmol/l)	*n*
Female						
	–	vehicle	0.88 ± 0.08	31	55.05 ± 4.3	28
	–	pGlu-Phe-Pro amide	2.21 ± 0.22**	8	99.65 ± 15.5**	8
	–	pGlu-Glu-Pro amide	1.50 ± 0.13**	7	48.15 ± 5.6	7
	–	pGlu-His-Pro amide (TRH)	2.14 ± 0.23**	6	100.38 ± 17.1**	6
	testosterone	vehicle	1.40 ± 0.17	12	33.27 ± 4.6	12
	testosterone	pGlu-Phe-Pro amide	1.58 ± 0.35	4	50.32 ± 4.5*	5
	testosterone	pGlu-Glu-Pro amide	1.60 ± 0.05	9	63.15 ± 8.1**	5
	testosterone	pGlu-His-Pro amide (TRH)	1.84 ± 0.19	5	78.69 ± 12.1**	5
Male						
	–	vehicle	1.20 ± 0.08	23	44.24 ± 2.3	23
	–	pGlu-Phe-Pro amide	2.30 ± 0.27**	8	58.47 ± 3.5**	8
	–	pGlu-Glu-Pro amide	1.40 ± 0.11	13	33.45 ± 5.3*	8
	–	pGlu-His-Pro amide (TRH)	2.05 ± 016**	10	65.00 ± 7.9**	10
	castration	vehicle	0.64 ± 0.04	6	43.28 ± 5.7	6
	castration	pGlu-Phe-Pro amide	1.59 ± 0.12**	5	86.72 ± 6.8**	5
	castration	pGlu-Glu-Pro amide	0.92 ± 0.11*	5	40.54 ± 4.5	5
	castration	pGlu-His-Pro amide (TRH)	2.20 ± 0.27**	5	85.39 ± 1.7**	5
	low potassium	vehicle	0.88 ± 0.06	15	48.10 ± 2.8	15
	low potassium	pGlu-Phe-Pro amide	1.90 ± 0.20**	10	134.25 ± 12.3**	10
	low potassium	pGlu-Glu-Pro amide	1.30 ± 0.30	10	57.31 ± 11.4	10
	low potassium	pGlu-His-Pro amide (TRH)	1.81 ± 0.11**	9	109.30 ± 11.3**	9

The experiments were carried out in female mice pretreated with testosterone, castrated male mice, or male mice on a low potassium diet.
* $P < 0.05$.
** $P < 0.01$.
n is the number of animals in each group.

ceived the same volume of vehicle. The peptides were given three times subcutaneously at 3 h intervals commencing at 0900 h in the morning and blood was taken 3 h after the final injection. The same protocol was used in the experiments in which female mice were treated with testosterone, male mice maintained on a low potassium diet, and in the experiments involving castration. Plasma was obtained by centrifugation and total T3 and total T4 levels were determined by radioimmunoassay (RIA) using commercial kits (Kodak Amerlex-M). For each determination, aliquots of plasma (50 μl) were analysed and the measurements were carried out in duplicate. To each sample was added 500 μl of ^{125}I-labeled T3 or ^{125}I-labeled T4 (approximately 50 000 cpm) and 500 μl of Amerlex-M antibody suspension (sheep anti-T3 or anti-T4). The solutions were mixed by vortexing and incubated for 60 min at 37°C for T3 or for 45 min at room temperature for T4. The suspensions were centrifuged at 4000 rpm (4°C) using a Beckman centrifuge (model J2-21) with a JA-14 rotor and adaptors for 3 ml tubes. The supernatants were removed by decantation and the pellets were counted using a Packard gamma counter (model D5002) with a Cobra II auto-gamma software package for calculations. The values obtained were assessed by comparison with appropriate T3 or T4 standards and are given in Table 1 in nmol/l. The sensitivity of the methods were > 0.15 nmol/l for T3, and > 4 nmol/l for T4. The cross-reactivity in the T3 assay at 50% standard binding was 0.3% for T4, and the cross-reactivity in the T4 assay was 12% for T3. Statistical analysis of the results was made with the aid of the computing program SPSS PC + . The Student's *t*-test was applied and changes were considered to be significant when P values of < 0.05 were obtained. The data are presented as mean values ± S.E.M.

3. Results

In our investigation of the effects produced by the TRH-like peptides pGlu-Glu-Pro amide and pGlu-Phe-Pro amide, and also by TRH, the peptides were administered subcutaneously in adult CD1 mice and the plasma levels of triiodothyronine (T3) and tetraiodothyronine (T4) were determined. pGlu-Phe-Pro amide and pGlu-His-Pro amide (TRH) produced major increases in the levels of T3 and T4 in the female mouse while pGlu-Glu-Pro amide produced an increase in T3 but no change in T4 (Fig. 1a and Table 1). In the male, the effects of these peptides on T3/T4 levels were less marked. In the case of pGlu-Glu-Pro amide no significant change in T3 levels could be detected; however there was a small but significant decrease in T4 levels (Fig. 2a, Table 1).

Measurement of the plasma levels of T3 and T4 in control untreated mice showed that the concentrations in the female were different from those in the male (Table 1). The levels of T3 in the male (1.20 ± 0.08 nmol/l) were significantly ($P < 0.01$) higher than in the female ($0.88 \pm$ 0.08 nmol/l). The T4 levels were higher in the female (55 ± 4.3 nmol/l) than in the male (44 ± 2.3 nmol/l).

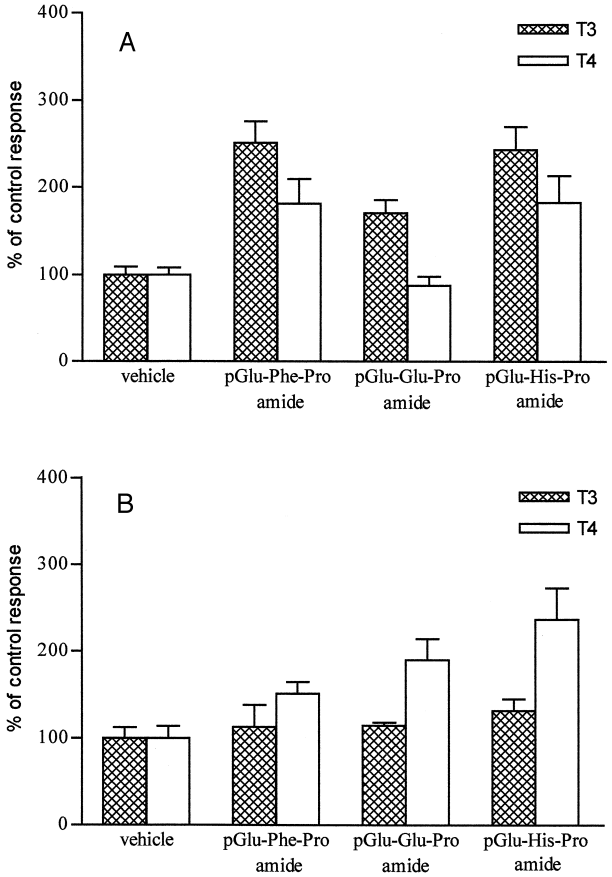

Fig. 1. Effects produced by subcutaneous administration of pGlu-Phe-Pro amide and pGlu-Glu-Pro amide on plasma T3 and T4 levels in CD1 adult female mice: (a) controls and (b) testosterone treated. Stippled bars = T3, open bars = T4. The results are presented as percentages of the respective control values. A statistical analysis of the results is shown in Table 1.

In female mice treated chronically with testosterone, the basal T3 levels were higher than in the untreated female mice while the T4 levels were slightly lower (Table 1). The basal levels of T3 in castrated male mice and males maintained on a potassium deficient diet were lower than in the corresponding untreated controls; in both cases no significant changes in T4 levels could be detected. In all the experiments the potencies of the TRH-like peptides in altering the levels of thyroid hormones were calculated by comparison with the corresponding control group.

In the testosterone treated female mice, negligible changes were observed in the T3 levels after administration of either the TRH-like peptides or TRH. The levels of T4, however, were increased by administration of each of the three peptides (Fig. 1b).

In the castrated male mice, administration of either pGlu-Phe-Pro amide or TRH led to substantial increases in T3 and T4 levels while pGlu-Glu-Pro amide produced a small increase in T3 but no change in T4 (Fig. 2b). In male mice on a low potassium diet, pGlu-Phe-Pro amide and TRH again produced substantial increases in T3 and T4

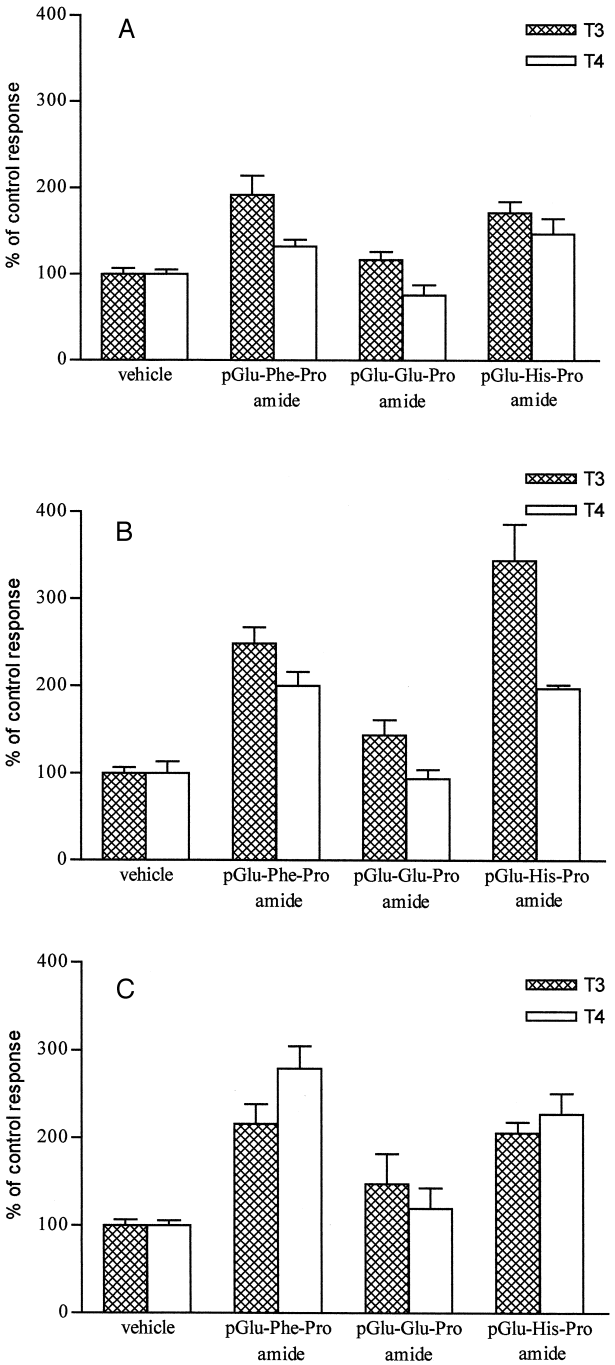

Fig. 2. Effects produced by subcutaneous administration of pGlu-Phe-Pro amide and pGlu-Glu-Pro amide on plasma T3 and T4 levels in CD1 adult male mice: (a) controls, (b) castrated and (c) low potassium diet. Stippled bars = T3, open bars = T4. The results are presented as percentages of the respective control values. A statistical analysis of the results is shown in Table 1.

levels in the plasma while pGlu-Glu-Pro amide had no significant effect on either T3 or T4 (Fig. 2c).

4. Discussion

The present study shows that the TRH-like peptides pGlu-Phe-Pro amide and pGlu-Glu-Pro amide, administered subcutaneously, possess the characteristic activity of TRH in raising the levels of T3 and T4 in the circulation. It is likely that this effect is mediated at the level of the pituitary through the release of thyroid stimulating hormone (TSH) which acts on the thyroid gland to release the thyroid hormones. In our experiments on the stimulated release of T3/T4, pGlu-Phe-Pro amide appeared to be as potent as TRH but pGlu-Glu-Pro amide was less active, which could be due to the presence of the negative charge on the γ-carboxyl group of glutamic acid at position 2. In any case it is clear that the nucleophilic character of the histidine residue of TRH is not necessary for the activity of this peptide in influencing thyroid status.

Our results on the in vivo activity of pGlu-Phe-Pro amide are consistent with the experiments of Sievertsson et al. (1972) who reported that synthetic pGlu-Phe-Pro amide can release TSH from the pituitary in vitro. It may be significant, however, that Ashworth et al. (1994) found that while pGlu-Glu-Pro amide stimulated the release of growth hormone and prolactin from GH3 pituitary cells, it did not displace ^{3}H-labelled methylhistidine-TRH or ^{3}H-labelled TRH from the membrane preparations. The TRH-like peptides, especially pGlu-Glu-Pro amide, may react preferentially with different receptors in the pituitary from those that react with TRH and this could account for the relatively low activity that is exhibited by pGlu-Glu-Pro amide in promoting the release of T3 and T4.

The mechanism involved in the increased release of T3/T4 by the TRH-like peptides could include not only an action at the level of the pituitary but also a direct action on the thyroid. In this context it has been shown that TRH has an inhibitory effect on dog thyroid in vitro (Delbeke et al., 1983; Iversen and Laurberg, 1985) and that administration of TRH to hyperthyroid patients with low levels of TSH resulted in a decrease in circulating thyroid hormones (Sato et al., 1995), suggesting that TRH can act on the thyroid in situ. The possibility that the TRH-like peptides, like TRH, also may act on the thyroid deserves consideration. Under the conditions of our experiments such an inhibitory action might be masked by the stimulatory effects induced by TRH and TRH-like peptides acting on the pituitary. However, it should be noted that the effect of pGlu-Glu-Pro amide on the male mouse was to produce a small but definite decrease in plasma T4 levels (Table 1). Since TRH-like peptides occur endogenously in the thyroid gland of many species (Del Rio-Garcia and Smyth, 1990; Rausell et al., 1998) and pituitary (Del Rio-Garcia and Smyth, 1990; Ashworth et al., 1991) the possibility exists that these peptides may fulfil physiological roles, either as paracrine agents within the thyroid or pituitary, or as endocrine factors signalling between the two glands.

It is of interest that the control female mice exhibited significantly lower basal T3 levels than the control male mice, which is in agreement with the report of Fukuda et al. (1975). However, the elevated levels of T3 observed in mice treated with TRH-like peptides were essentially the

same in the male as they were in the female and consequently the TRH-like peptides appeared to produce a greater effect in the female than in the male. In addition, we found that castration of male mice, which is known to lead to reduced levels of circulating testosterone, led to lower basal levels of T3 than in the untreated controls, pointing to an influence of testosterone on the hypothalamic–hypophyseal thyroid axis. Similarly, treatment of the female mice with testosterone led to elevated T3 levels. After castration of the male or testosterone treatment of the female, administration of the TRH-like peptides (and TRH) resulted in essentially the same elevated T3 levels that were observed in the controls treated with these peptides. Thus, castration of the male mouse appears to render it more sensitive to the activity of TRH-like peptides whereas testosterone treatment of the female results in reduced sensitivity.

The effects of potassium deprivation on the levels of plasma testosterone are well documented (Sanchez-Capelo et al., 1993). Male mice maintained on a diet deficient in potassium exhibit low levels of circulating testosterone and consequently may be expected to show a greater sensitivity to TRH and TRH-like peptides. This was in fact observed: the plasma T3 levels in potassium deprived male mice were increased by administration of both pGlu-Phe-Pro amide and pGlu-Glu-Pro amide (and by TRH) to a greater extent than was observed in the male mouse on the standard laboratory diet. This supports the view that testosterone influences the levels of T3 in the circulation and thus modifies the sensitivity of the mouse in its response to challenge by TRH-like peptides.

The main finding in the present study is that the TRH-like peptides pGlu-Phe-Pro amide and pGlu-Glu-Pro amide, which occur naturally in the thyroid, possess the ability to increase thyroid hormone levels in vivo. Their potencies are sensitive to testosterone, present endogenously or administered exogenously.

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