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Physiology

Processing of thyrotropin-releasing hormone prohormone (pro-TRH) generates a biologically active peptide, prepro-TRH-(160–169), which regulates TRH-induced thyrotropin secretion

(hypothalamo–pituitary–thyroid axis/neurosecretion)

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ABSTRACT **Rat thyrotropin-releasing hormone (TRH) prohormone contains five copies of the TRH progenitor sequence Gln-His-Pro-Gly linked together by connecting sequences whose biological activity is unknown. Both the predicted connecting peptide prepro-TRH-(160–169) (Ps4) and TRH are predominant storage forms of TRH precursor-related peptides in the hypothalamus. To determine whether Ps4 is co-released with TRH, rat median eminence slices were perifused *in vitro*. Infusion of depolarizing concentrations of KCl induced stimulation of release of Ps4- and TRH-like immunoreactivity. The possible effect of Ps4 on thyrotropin release was investigated *in vitro* using quartered anterior pituitaries. Infusion of Ps4 alone had no effect on thyrotropin secretion but potentiated TRH-induced thyrotropin release in a dose-dependent manner. In addition, the occurrence of specific binding sites for ^{125}I-labeled Tyr-Ps4 in the distal lobe of the pituitary was demonstrated by binding analysis and autoradiographic localization. These findings indicate that these two peptides that arise from a single multifunctional precursor, the TRH prohormone, act in a coordinate manner on the same target cells to promote hormonal secretion. These data suggest that differential processing of the TRH prohormone may have the potential to modulate the biological activity of TRH.**

Thyrotropin-releasing hormone (TRH) is a tripeptide amide (<Glu-His-Pro-NH_2, where <Glu is pyroglutamic acid), which was originally isolated from ovine and porcine hypothalami and characterized for its ability to stimulate thyrotropin (TSH) secretion (1, 2). Although TRH was the first hypophysiotropic neuropeptide to be characterized, the sequence of its precursor has only recently been elucidated (3–5). Like other biologically active peptides that contain multiple closely related sequences (6–8) or homologous sequences (9–12), TRH is synthesized as a large precursor protein that contains multiple copies of the TRH progenitor sequence Gln-His-Pro-Gly, each sequence flanked by paired basic amino acid residues. Proteolytic cleavage at these loci can yield five copies of TRH, which account for 8% of the total molecular weight of the precursor, and seven cryptic peptides including two peptides derived from the N-terminal flanking sequence, four connecting sequences intercalated among the five TRH progenitor sequences, and a C-terminal flanking sequence (Fig. 1). In the rat hypothalamus, the TRH precursor undergoes nearly complete processing to generate authentic TRH and TRH-free connecting sequences (14, 15). However, C-terminally extended forms of TRH, resulting from incomplete processing of the precursor molecule, have been detected in faint amounts (16).

The biological function(s) of cryptic peptides deriving from precursor proteins are currently a matter of speculation. It has been proposed that they may participate in the processing and/or intracellular routing of the prohormone (17). Alternatively, the connecting peptides may exhibit biological activities distinct from or coordinated with their related regulatory peptide. For instance, posttranslational processing of the multifunctional precursor protein proopiomelanocortin generates a 27-amino acid peptide called γ_3-melanotropin, which potentiates adrenocorticotropin-evoked glucocorticoid secretion (18). Likewise, neuropeptide K and substance P, two takykinins derived from the same precursor, exert synergistic stimulation on salivation (19).

We have synthesized a peptide corresponding to the pro-TRH-intervening sequence prepro-TRH-(160–169) (Ps4) to investigate the possible functions of this cryptic peptide. By combining HPLC analysis and radioimmunoassay detection, we have observed that Ps4 and TRH are authentic storage forms of the TRH precursor in the rat hypothalamus (15). In addition, immunocytochemical studies at the electron microscopic level have shown that pro-TRH-connecting peptides are sequestered within dense core secretory vesicles in hypothalamic neurons and fibers (20), suggesting that these peptides are likely released with TRH in the external zone of the median eminence. In the present study, we demonstrate that the connecting peptide Ps4 is actually co-released with TRH by hypothalamic slices and we document the occurrence of specific binding sites for ^{125}I-labeled Tyr-Ps4 in the anterior pituitary. In addition, we show that Ps4 potentiates the stimulatory effect of TRH on TSH release by rat pituitary glands *in vitro*.

EXPERIMENTAL PROCEDURES

Animals. Adult male Wistar rats, weighing 200–280 g, were used. They were housed in cages (five rats per cage) under controlled conditions of light (lights-on time, 0700–1900) and temperature (21°C). The animals were given laboratory chow and water ad libitum.

Perifusion Procedures. Rats were killed by decapitation at a time between 0900 and 1000, their brains were exposed, and the medial-basal hypothalamus was removed. Hypothalamic slices (400 μm thick) from four animals were transferred into a siliconized glass chamber delimited by two Teflon pestles

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Abbreviations: TRH, thyrotropin-releasing hormone; Ps4, prepro-TRH-(160–169); TSH, thyrotropin.

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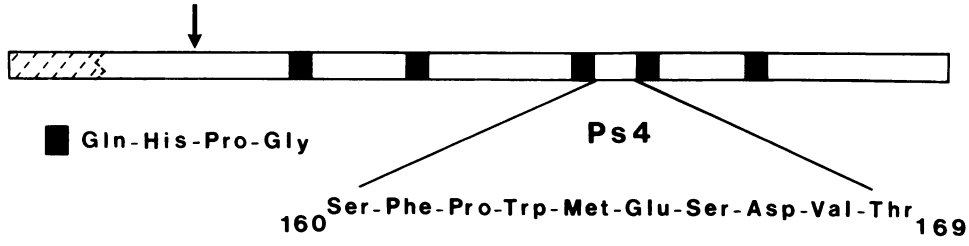

FIG. 1. Schematic representation of the structure of rat prepro-TRH (4). The prepro-TRH-(1–24) sequence (hatched bar) is the putative signal peptide sequence (13). Positions of the TRH progenitor sequences, Gln-His-Pro-Gly, which are flanked by pairs of basic amino acid residues, are shown (solid bars). An additional potential cleavage site located in the N-terminal flanking sequence is indicated (arrow). The sequence of Ps4 is shown.

as described (21). The tissue slices were perifused at constant flow rate (8.4 ml/hr) and constant temperature (37°C) with Krebs–Ringer's bicarbonate buffer (pH 7.4). The effluent was collected in 10-min fractions of 1.4 ml and the concentrations of Ps4 and TRH were determined by radioimmunoassay in duplicate, as described (15, 22). To induce depolarization, KCl (70 mM) pulses of 15-min duration were administered, the concentration of NaCl being proportionally lowered to keep a constant osmolarity. For chromatographic analysis of the effluent perifusate, five fractions were pooled (7 ml), prefiltered on a Sep-Pak C_{18} cartridge, and subjected to reverse-phase HPLC on an Orpegen RP-7S-300 column (0.4 × 15 cm) using isocratic conditions [22% acetonitrile/0.1% trifluoroacetic acid/77.9% H_2O (vol/vol)].

Quartered rat pituitaries were perifused with medium 199 (GIBCO) as described (23) and the amount of TSH released in the effluent perifusate was measured by radioimmunoassay. A 6-min pulse of TRH (10^{-8} M) was infused alone or during prolonged administration of Ps4 (10^{-10} to 10^{-5} M).

Radioreceptor Assay. Ps4 and Tyr-Ps4 were synthesized as described (15). Tyr-Ps4 was radioiodinated using chloramine T and the labeled peptide was purified by reverse-phase HPLC as described (15). The specific activity of the tracer used for receptor studies was 2000 Ci/mmol (1 Ci = 37 GBq).

Fifty anterior pituitary glands were homogenized with 50 mM Tris-Cl/0.32 M sucrose, pH 7.4, in a Potter–Elvehjem homogenizer and the homogenate was centrifuged at 600 × *g* for 10 min. The supernatant was washed by two cycles of Teflon–glass homogenizer and centrifuged (40,000 × *g* for 20 min) at 4°C in the same buffer. The resulting pellet was gently resuspended in 20 ml of 50 mM Tris Cl (pH 7.4) and stored at −80°C. The final protein concentration of the membrane-enriched preparation was 0.27 mg/ml, as determined by the method of Lowry *et al.* (24) using bovine serum albumin as standard. Binding assay was performed as described (25) with some modifications. ^{125}I-labeled Tyr-Ps4 was incubated with 27 μg of membrane proteins in 50 mM Tris Cl (pH 7.4) containing 0.1% bovine serum albumin and 0.01% bacitracin, in a total volume of 300 μl. Incubations were performed for 120 min at 24°C and the bound radioactivity was separated by filtration through 0.1% polyethylenimine-coated Whatman GF/C glass fiber filters. The filters were washed twice with 10 ml of ice-cold 50 mM Tris Cl (pH 7.4) containing 0.1% bovine serum albumin. After drying, the radioactivity retained on the filter was quantified by γ counting. Nonspecific binding was determined in the presence of 1 μM unlabeled Tyr-Ps4.

Degradation of Peptides by Pituitary Membranes. Synthetic Ps4-related peptides were incubated with rat pituitary membranes (27 μg of protein per tube) at 24°C for 120 min. The samples were then centrifuged (40,000 × *g*; 20 min; 4°C) and the supernatants were immediately analyzed by reverse-phase HPLC using a linear gradient of acetonitrile (0–60% in 60 min).

The amino acid compositions of the peptides separated on HPLC were determined after hydrolysis (6 M HCl in sealed tubes at 105°C for 24 hr). The released residues were treated by phenylisothiocyanate and analyzed by HPLC as described (26).

Autoradiographic Study. Unfixed rat pituitaries were sliced on a cryostat at 20 μm. The slices were preincubated for 10 min in 50 mM Tris Cl (pH 7.4) containing 0.1% bovine serum albumin and 0.01% bacitracin and then further incubated with 0.2 nM ^{125}I-labeled Tyr-Ps4 for 120 min at 24°C as described (27). The slices were washed for four 30-s periods with ice-cold buffer and dried. To visualize nonspecific binding, slices were incubated with 0.2 nM ^{125}I-labeled Tyr-Ps4 in the presence of 1 μM Tyr-Ps4. The slices were apposed to LKB Ultrofilms for 48 hr and the corresponding tissue sections were stained with cresyl violet (Nissl stain) for histological examinations.

RESULTS

Release of Ps4-Related Peptides by Hypothalamic Slices. The rate of spontaneous efflux of Ps4-like immunoreactivity remained constant and very low during perfusion experiments (Fig. 2*A*). The basal release (10 pg per hypothalamus per 10 min) represented approximately 0.12% of total hypothalamic Ps4-like immunoreactivity content per min. Infusion of depolarizing concentrations of KCl induced a robust and transient stimulation of Ps4- and TRH-like immunoreactivity (Fig. 2*A*). The ratio of released TRH- vs. Ps4-like immunoreactivity was approximately 4:1 (on a molar basis). Serial dilutions of the effluent perifusate gave displacement curves that were parallel to the standard curve obtained with synthetic Ps4 (Fig. 2*B Inset*). In addition, isocratic reverse-phase HPLC analysis of the effluent perifusate resolved one major immunoreactive species that exhibited the same retention time as synthetic Ps4 (Fig. 2*B*).

Localization and Characterization of Ps4-Binding Sites in Rat Pituitary. By using ^{125}I-labeled Tyr-Ps4, autoradiographic studies revealed the presence of a high density of Ps4-binding sites in the anterior lobe of the pituitary (Fig. 3*A*). Incubation of pituitary slices with ^{125}I-labeled Tyr-Ps4 in the presence of an excess of synthetic Tyr-Ps4 (10^{-6} M) totally prevented binding (Fig. 3*B*). Cresyl violet staining of the pituitary sections used for autoradiography revealed that ^{125}I-labeled Tyr-Ps4 binding sites were exclusively located in the anterior lobe, while the neural lobe and the intermediate lobe were not labeled (Fig. 3*C*). Characterization of these ^{125}I-labeled Tyr-Ps4 binding sites using membrane-enriched preparations from rat anterior pituitary showed that binding of the radioligand was saturable (Fig. 4*A*). Scatchard plot analysis of saturation experiments revealed the occurrence of a single population of binding sites with a binding capacity of 702 fmol/mg of membrane proteins and a K_d value of 0.18 nM. Nonspecific binding was relatively low (7% at the K_d). Competition experiments showed that specific binding was

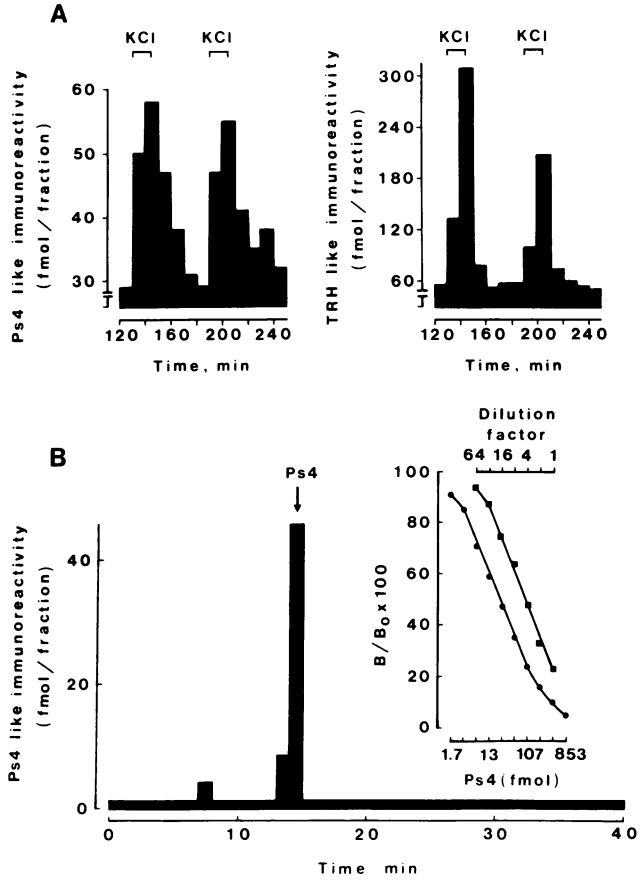

FIG. 2. Release of Ps4 and TRH from rat mediobasal hypothalamus *in vitro*. (*A*) Effect of two consecutive depolarizing pulses of KCl (70 mM) on release of Ps4 and TRH by hypothalamic slices. The concentrations of Ps4 and TRH were measured by radioimmunoassay in duplicate. (*B*) Reverse-phase HPLC analysis of effluent medium collected from a perifusion column. Forty 0.75-ml fractions were collected, lyophilized, and radioimmunoassayed in duplicate. The arrow indicates the elution position of synthetic Ps4. (*Inset*) Competition curves obtained with synthetic Ps4 (circles) or serial dilutions of effluent collected from a perifusion column (squares). B and B_0 are the amounts of antibody-bound tracer, in the presence or absence of unlabeled peptide, respectively.

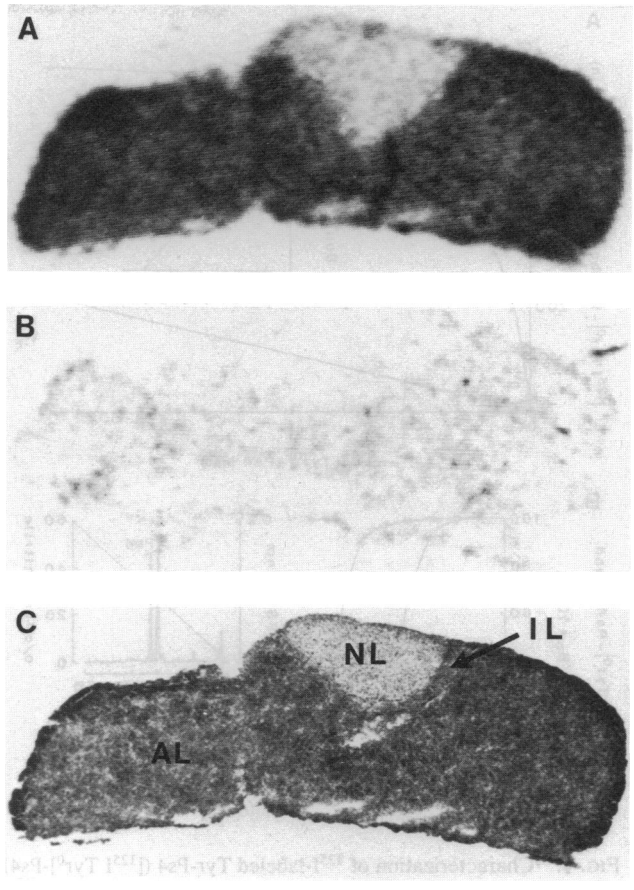

FIG. 3. Distribution of ^{125}I-labeled Tyr-Ps4 binding sites in the rat pituitary. (*A*) Autoradiographic localization of binding sites in coronal sections of rat pituitary. (*B*) Nonspecific binding in the presence of excess (1 μM) unlabeled Tyr-Ps4. (*C*) Cresyl violet staining of the section used for autoradiography in *A*, showing that only the anterior lobe (AL) contains ^{125}I-labeled Tyr-Ps4 binding sites but both the intermediate lobe (IL) and neural lobe (NL) are devoid of binding sites.

displaced by both Tyr-Ps4 and Ps4 in a dose-dependent manner (K_i values were 1.05 nM and 0.43 μM, respectively). In contrast, various neuropeptides, including TRH, prepro-TRH-(178–199), the hypophysiotropic neurohormones growth hormone-releasing factor, and corticotropin-releasing factor, or the proopiomelanocortin-derived peptides α-melanotropin and β-endorphin, did not induce any displacement of membrane bound ^{125}I-labeled Tyr-Ps4 (Fig. 4*B*). Reverse-phase HPLC analysis of the peptides after a 120-min incubation with pituitary membranes revealed that ^{125}I-labeled Tyr-Ps4 and Tyr-Ps4 were intact (data not shown) but Ps4 was partly converted into a less hydrophobic species (Fig. 4*B Inset*). This degradation product appeared to correspond to Des-Ser^1,Phe^2-Ps4, as revealed by analysis of its amino acid composition.

Effect of Ps4 on TSH Release. Administration of Ps4 had no effect on the spontaneous secretion of TSH but induced a marked potentiation of TRH-evoked TSH release (Fig. 5*A*). In fact Ps4 both increased the magnitude of the acute response observed during TRH administration and prolonged the duration of the response of pituitary thyrotropes to TRH. Similar experiments were performed with graded doses of Ps4 (Fig. 5*B*). A significant stimulation of TRH-evoked TSH secretion was already observed when equimolar concentrations of TRH and Ps4 (10^{-8} M) were administered. Half-maximal potentiation occurred at 7.5×10^{-8} M: at this concentration Ps4 caused approximately a 2-fold augmentation of TRH-induced TSH release. The effect of Ps4 was maximal at 10^{-6} M. The specificity of the potentiating action of Ps4 was investigated by using the nonapeptide oxytocin, which had no effect on TRH-induced TSH release. In contrast, the analog Tyr-Ps4 was approximately as active as Ps4 in potentiating the action of TRH (data not shown).

DISCUSSION

Previous studies have shown that, in the rat median eminence, processing of the TRH precursor nearly reaches completion and generates TRH-free connecting peptides (14, 15), including Ps4. In the present investigation, we demonstrate that medial basal hypothalamic slices release authentic Ps4 and that K^+-induced depolarizations stimulate secretion of both TRH and Ps4, suggesting that *in vivo* the two peptides must be released together under physiological stimuli. This hypothesis is supported by immunohistochemical studies that show that TRH-intervening sequences are sequestered in dense core vesicles contained in nerve fibers terminating in the external zone of the median eminence (20). These data strongly suggest that Ps4, as well as TRH, can reach the anterior pituitary by the portal system.

To investigate the possible site of action of Ps4 at the pituitary level, we have used iodinated Tyr-Ps4 as a radioli-

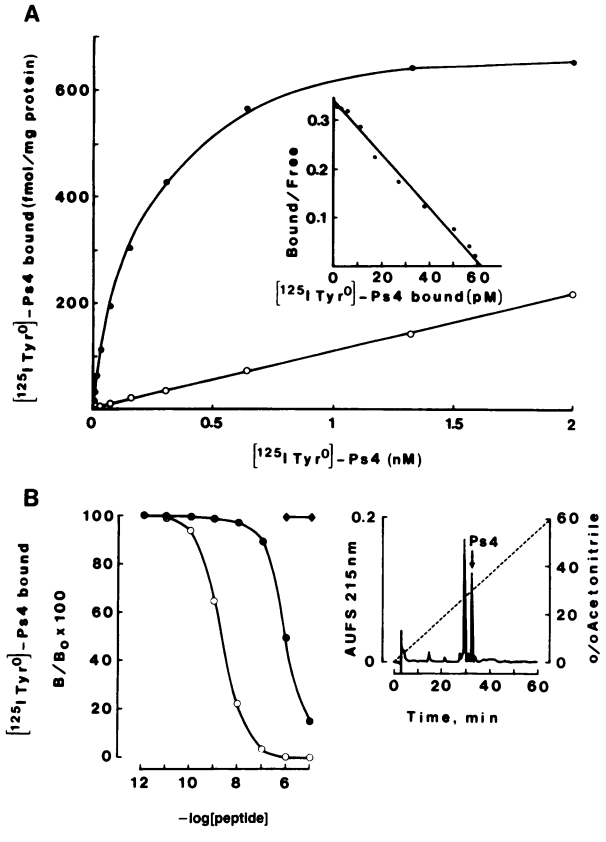

FIG. 4. Characterization of ^{125}I-labeled Tyr-Ps4 ([^{125}I Tyr^0]-Ps4) binding sites in the rat pituitary. (*A*) Saturation isotherm of ^{125}I-labeled Tyr-Ps4 binding and the corresponding Scatchard plot (K_d = 0.18 nM; B_{max} = 702 fmol/mg of protein). Specific (solid circles) and nonspecific binding (open circles) are represented. Nonspecific binding was determined by incubating membranes with the radioligand in the presence of 10^{-6} M Tyr-Ps4. (*B*) Inhibition of ^{125}I-labeled Tyr-Ps4 binding by Tyr-Ps4 (open circles), Ps4 (solid circles), and various neuropeptides including substance P, growth hormone-releasing hormone, corticotropin-releasing factor, oxytocin, vasopressin, neurotensin, TRH, prepro-TRH-(178–199), α-melanotropin, deacetyl α-melanotropin, and β-endorphin (solid rhombus). The inhibition constants (K_i) were calculated as: $K_i = IC_{50}/[1 + (L/K_d)]$ where L is the free radioligand concentration in equilibrium with IC_{50} of unlabeled ligand (28). (*Inset*) HPLC profile of an excess of Ps4 (20 μg) after a 120-min incubation with 27 μg of pituitary membrane proteins showing the extent of degradation of Ps4. The arrow indicates the elution position of synthetic (intact) Ps4. The major peak eluting at 30 min was identified by amino acid analysis as Des-Ser^1,Phe^2-Ps4. AUFS, arbitrary units, full scale.

gand. The autoradiographic data reported herein reveal that the anterior lobe of the pituitary contains a high density of Ps4 binding sites whereas the posterior lobe is totally devoid of sites. The concentration of ^{125}I-labeled Tyr-Ps4 binding sites in the adenohypophysis was in the same range as that reported (29) for TRH receptors. Displacement experiments revealed an apparent discrepancy between the K_i values measured with Tyr-Ps4 and Ps4 (nanomolar and micromolar range, respectively). However, incubation of synthetic peptides with rat pituitary membranes indicated that Ps4 undergoes rapid degradation when exposed to proteases but the analogue Tyr-Ps4 is far more resistant to enzymatic cleavage. The degraded form of Ps4 corresponds to the sequences Des-Ser^1,Phe^2-Ps4, as determined by amino acid analysis, suggesting that inactivation of Ps4 by pituitary cell membranes may result from the action of a dipeptidyl aminopeptidase. These observations may provide a clue for the design of stable agonists of Ps4.

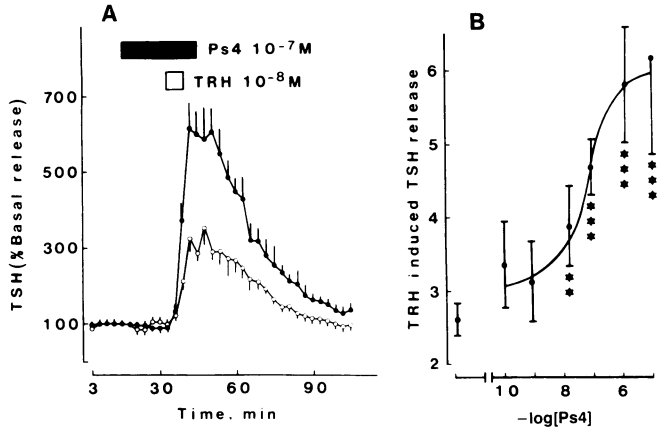

FIG. 5. Effect of synthetic Ps4 on TSH release by pituitary fragments. (*A*) Perifusion profiles showing the effect of a short pulse of TRH (10^{-8} M) alone (open circles) or TRH (10^{-8} M) during prolonged infusion of Ps4 (10^{-7} M) (solid circles). Ps4 did not modify the spontaneous release of TSH, but significantly potentiated TRH-evoked TSH release. (*B*) Effect of graded concentrations of Ps4 (10^{-10} M to 10^{-5} M) on TRH-evoked TSH release. The dose–response curve was constructed from a series of experiments similar to that shown in *A*. The concentration of TRH was 10^{-8} M throughout. Each point was calculated as the net increase of TSH release during the first 15-min of the initial burst of release. The asterisks indicate that the values were significantly different from the response obtained with TRH alone: **, $P < 0.01$; ***, $P < 0.001$.

The occurrence of a high density of Ps4 binding sites in the rat adenohypophysis prompted us to investigate the possible effects of this peptide on pituitary hormone secretion. We found that Ps4 does not affect TSH secretion but potentiates the action of TRH on TSH release. Previous data have shown that the precursor for gonadotropin-releasing hormone contains, in addition to the decapeptide gonadotropin-releasing hormone, a 56-amino acid peptide that inhibits prolactin and stimulates gonadotropin secretion from rat pituitary cells (30). Concurrently, it has been demonstrated that distinct hypothalamic peptides may cooperate, in a synergistic manner, in the regulation of pituitary hormone secretion. For example, vasopressin (31, 32) and oxytocin (33, 34) potentiate the action of corticotropin-releasing factor on pituitary corticotrophs. However, whereas these latter studies showed that neuropeptides originating from distinct precursor molecules fulfill coordinate physiological roles, the present results provide evidence that two peptides derived from a single precursor may act in a synergistic manner on the same target cells to modulate hormone release. Thus our data suggest that differential processing of the TRH precursor may constitute an important regulatory point in modulating the biological potency of TRH-connecting sequences.

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