

Interaction between substance P and TRH in the control of prolactin release

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Abstract

Substance P (SP) may participate as a paracrine and/or autocrine factor in the regulation of anterior pituitary function. This project studied the effect of TRH on SP content and release from anterior pituitary and the role of SP in TRH-induced prolactin release. TRH (10^{-7} M), but not vasoactive intestinal polypeptide (VIP), increased immunoreactive-SP (ir-SP) content and release from male rat anterior pituitary *in vitro*. An anti-prolactin serum also increased ir-SP release and content. In order to determine whether intrapituitary SP participates in TRH-induced prolactin release, anterior pituitaries were incubated with TRH (10^{-7} M) and either WIN 62,577, a specific antagonist of the NK1 receptor, or a specific anti-SP serum. Both WIN 62,577 (10^{-8} and 10^{-7} M) and the anti-SP serum (1:250) blocked TRH-induced prolactin

release. In order to study the interaction between TRH and SP on prolactin release, anterior pituitaries were incubated with either TRH (10^{-7} M) or SP, or with both peptides. SP (10^{-7} and 10^{-6} M) by itself stimulated prolactin release. While 10^{-7} M SP did not modify the TRH effect, 10^{-6} M SP reduced TRH-stimulated prolactin release. SP (10^{-5} M) alone failed to stimulate prolactin release and markedly decreased TRH-induced prolactin release. The present study shows that TRH stimulates ir-SP release and increases ir-SP content in the anterior pituitary. Our data also suggest that SP may act as a modulator of TRH effect on prolactin secretion by a paracrine mechanism.

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Introduction

Substance P (SP), a neuropeptide belonging to the tachykinin family, is found in most tissues, and produces a broad range of cellular responses. SP exerts its effects through a specific receptor in a G protein-linked family of receptors. It enhances polyphosphoinositide breakdown and increases intracellular free calcium levels (Mau *et al.* 1990, Mau & Saermark 1991). In the anterior pituitary, the preferred SP receptor, a neurokinin-1 (NK1) subtype of the tachykinin receptor family, is found exclusively in lactotrophs and gonadotrophs (Larsen *et al.* 1992).

SP is synthesized (Jessop *et al.* 1992) and released (Seilicovich *et al.* 1990, Arita *et al.* 1993) by the anterior pituitary. SP was found to be localized only in somatotrophs and thyrotrophs of male rats (Brown *et al.* 1991, Arita *et al.* 1994). Also SP-like immunoreactive innervation is present in the anterior pituitary, distributed around secretory cells such as lactotrophs (Liu *et al.* 1996) and corticotrophs (Ju *et al.* 1991) and around blood vessels (Mikkelsen *et al.* 1989).

SP content and preprotachykinin mRNA vary with age and sex of the animals; it is higher in male than in female

rats (Yoshikawa & Hong 1983, Jessop *et al.* 1992). SP content in the anterior pituitary is regulated by several circulating hormones (De Palatis *et al.* 1985, Jonnassen *et al.* 1987, Aronin *et al.* 1988, O'Halloran *et al.* 1990, Jones *et al.* 1994).

SP modulates several anterior pituitary hormones (Jessop *et al.* 1992). SP stimulates *in vitro* prolactin release from the anterior pituitary (Vijayan & McCann 1979, Seilicovich *et al.* 1993), while the blockade of intrapituitary SP with specific SP antibodies decreases basal prolactin release (Afione *et al.* 1990a).

Thyrotropin-releasing hormone (TRH) is a hypothalamic neuropeptide that increases thyrotropin (TSH) and prolactin secretion. TRH is synthesized not only in the hypothalamus but also in a subpopulation of somatotrophs in the anterior pituitary (Bruhn *et al.* 1994).

Several studies have demonstrated the modulatory role of some pituitary peptides on hypothalamic neurotransmitters and neuropeptides that regulate anterior pituitary hormone secretion (Peillon *et al.* 1997). Interactions between neurotransmitters and SP or vasoactive intestinal polypeptide (VIP) at pituitary level (Afione *et al.* 1990a,b, Duvilanski *et al.* 1990, Apfelbaum 1998), and also

between VIP and TRH (Balsa *et al.* 1996) have been described.

Since SP has a paracrine effect on prolactin secretion, the aim of this study was to ascertain whether anterior pituitary SP is involved in the effect of TRH on prolactin release. Therefore, we designed our experiments in order to study: (1) the influence of SP blockade on the effect of TRH on prolactin release; (2) the effect of TRH on anterior pituitary SP release and content; and (3) the interaction between TRH and SP in the control of prolactin release.

Materials and Methods

Male Wistar rats (200–250 g) were kept in controlled conditions of light (12 h light : 12 h darkness) and temperature (21–24 °C). Lab chow and water were supplied and animals were allowed to feed *ad libitum*. The rats were kept according to the NIH Guide for the Care and Use of Laboratory Animals. Rats were killed by decapitation and the anterior pituitary lobes removed, then placed in Krebs–Ringer bicarbonate buffer, pH 7.4, containing 10 mM glucose, 25 mM Hepes, 0.1 mM bacitracin, 0.1% ascorbic acid and 0.1% bovine serum albumin (KRB buffer).

All drugs were purchased from Sigma (St Louis, MO, USA), except TRH, SP and VIP (Peninsula Lab. Inc., Belmont, CA, USA), and WIN 62,577 (Research Biochemical Int., Natick, MA, USA). Rat prolactin and NIDDK anti-prolactin serum (S-9) were kindly supplied by the National Hormone and Pituitary Program (Torrance, CA, USA).

Rabbit anti-SP serum was prepared, characterized and tested as previously reported (Debeljuk *et al.* 1988). This anti-serum showed no cross-reactivity with other mammalian neuropeptides such as neurokinin A, neurokinin B, VIP, neuromedin B and neurotensin when these peptides were tested at doses 5000–10 000-fold higher than those of SP.

Dynamic incubation of anterior pituitary glands. Pulse-chase experiments

Five pituitaries were placed into a small perforated basket and immersed in a tube containing 1 ml of KRB buffer and pre-incubated for 30 min at 37 °C, in an atmosphere of 95% O₂–5% CO₂, with constant shaking of 60 cycles per min. Basal release was then determined by incubating the baskets for two periods of 15 min in fresh KRB buffer. Afterwards, the baskets were transferred to a tube containing TRH (10⁻⁷ M) and incubated for 5 min (TRH pulse). Finally, post-stimulus release was assessed by incubating the baskets for two periods of 15 min in KRB buffer. Controls were run in parallel with KRB buffer only. The media were immediately acidified, frozen on

dry ice and processed for SP determination. An aliquot of each medium was separated before acidification and stored at –20 °C until prolactin determination.

Static incubation of anterior pituitary glands

Three pituitaries per tube were used to assay SP and prolactin release in each incubation, and only one when prolactin release alone was studied. The pituitaries were pre-incubated for 60 min in 1 ml KRB buffer. Afterwards, the tissue was incubated for another 60 min, unless otherwise indicated, in 1 ml of fresh KRB buffer containing the different drugs studied. Controls were incubated with medium alone or vehicle or normal rabbit serum (NRS). At the end of the incubation period, media were aspirated. Media and tissues were immediately acidified, frozen on dry ice and processed for SP determination. An aliquot of medium was separated before acidification, and stored at –20 °C until prolactin determination. WIN 62,577 is a non-peptide NK1 receptor antagonist that binds to rat brain homogenates with K_i values of 21 nM (Appel *et al.* 1991). WIN 62,577 was dissolved in DMSO and diluted in KRB buffer. A final concentration of 0.02% DMSO had no effect on basal prolactin release.

SP determination

Media and tissues were heated at 100 °C for 5 min in 1 M acetic acid. The tissues were then homogenized and both media and tissues centrifuged at 14 500 r.p.m. for 20 min. The supernatants were lyophilized and stored at –70 °C until SP determination. SP was measured by double antibody radioimmunoassay with ¹²⁵I-SP as tracer, as previously described (Seilicovich *et al.* 1990). The intra- and interassay coefficients of variation were under 10%. Immunoreactive SP (ir-SP) was expressed as ng/mg protein. Protein concentration of tissue pellets was determined by Lowry's method.

Prolactin determination

Prolactin was measured in the incubation medium by a double antibody radioimmunoassay, with kits provided by the National Hormone and Pituitary Program. Results were expressed in terms of rat prolactin RP-3 standard. The intra- and interassay coefficients of variation were less than 9%.

Statistical analysis

Results were expressed as means ± s.e.m.s and evaluated by the Student *t*-test or by one- or two-way analysis of variance (ANOVA) followed by Student–Newman–Keuls multiple comparison test for unequal replicates or

Table 1 Effect of WIN 62,577 on basal and SP-induced prolactin release. Anterior pituitaries were incubated for 60 min in the presence of increasing concentrations of WIN 62,577 (WIN), a specific SP receptor antagonist, with or without SP. Data are means \pm S.E.M. ($n=6$)

	Prolactin ($\mu\text{g}/\text{mg}$ protein)	
	No SP added	SP (10^{-6} M)
Control	1.35 \pm 0.10	1.71 \pm 0.09*
WIN (10^{-8} M)	1.47 \pm 0.11	1.47 \pm 0.08
WIN (10^{-7} M)	1.27 \pm 0.05	1.32 \pm 0.08†
WIN (10^{-6} M)	0.82 \pm 0.08†	—

* $P<0.01$ vs respective control without SP, † $P<0.01$ vs respective control without WIN. Statistical significance was determined by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test.

Dunnett's test. The differences between groups were considered significant if $P<0.05$. Results were confirmed by at least three independent experiments.

Results

Effect of the blockade of intrapituitary SP on TRH-induced prolactin release

WIN 62,577 (10^{-6} M), a specific antagonist of the NK1 receptor, inhibited basal prolactin release. Lower concentrations of the receptor antagonist (10^{-8} – 10^{-7} M) had no effect on prolactin release but significantly reduced SP-induced prolactin release (Table 1) and also blocked the stimulatory effect of TRH (Fig. 1). A specific anti-SP serum (1:250) did not modify basal prolactin release from anterior pituitaries incubated for 20 min but significantly decreased basal prolactin release after 60 min (Fig. 2A, B)

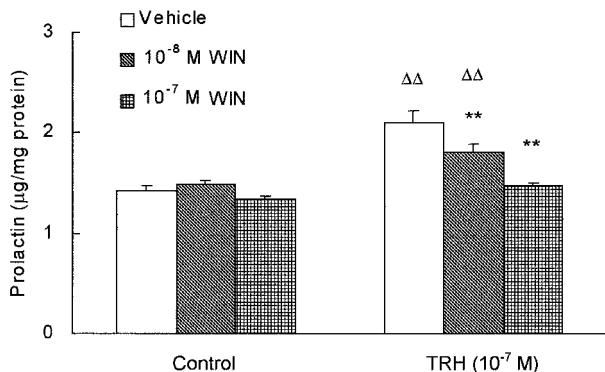


Figure 1 SP receptor antagonist blocks TRH-induced prolactin release. Anterior pituitaries were incubated for 60 min with 10^{-7} M TRH and/or WIN 62,577 (WIN). Bars represent means \pm S.E.M.s ($n=6$). Statistical significance was determined by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test. ** $P<0.01$ vs respective control without WIN. $\Delta\Delta P<0.01$ vs respective control without TRH.

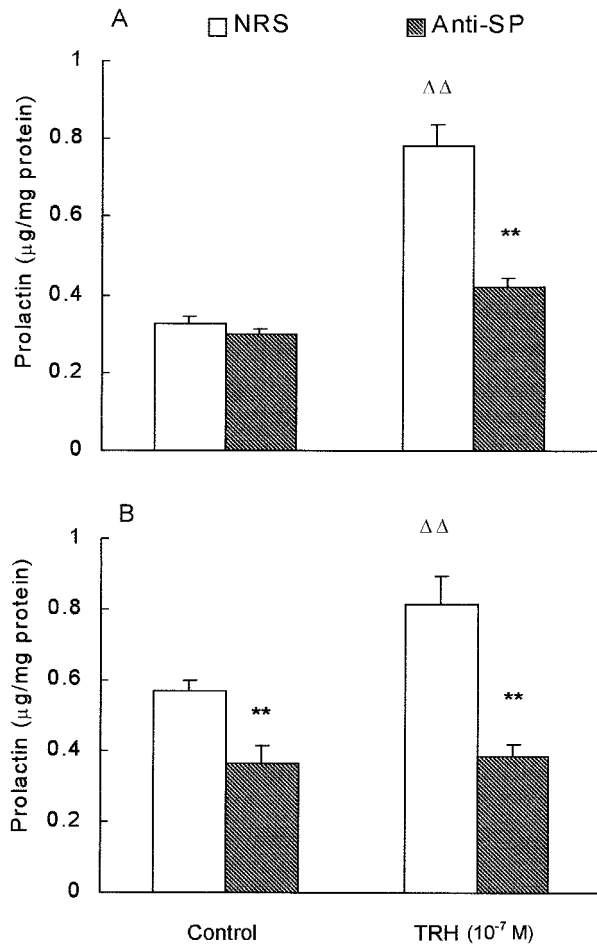


Figure 2 Anti-SP serum blocks TRH-induced prolactin release. Anterior pituitaries were incubated for 20 (A) and 60 (B) min with 10^{-7} M TRH and anti-SP serum (Anti-SP, final dilution 1:250). Controls were incubated with normal rabbit serum (NRS, final dilution 1:250). Bars represent means \pm S.E.M.s ($n=5$). Statistical significance was evaluated by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test. ** $P<0.01$ vs respective control without Anti-SP. $\Delta\Delta P<0.01$ vs respective control without TRH.

and inhibited TRH-induced prolactin release in both incubation periods (Fig. 2A, B).

Effect of TRH on ir-SP release

Since intrapituitary SP seems to be involved in the stimulatory effect of TRH on prolactin release, we examined whether TRH affects SP release. TRH (10^{-7} M) stimulated ir-SP release and increased tissue content of ir-SP in anterior pituitary incubated in static conditions (Fig. 3). TRH also stimulated prolactin release (control: 0.823 ± 0.053 $\mu\text{g}/\text{mg}$ protein; 10^{-7} M TRH: 1.817 ± 0.111 , $n=7$, $P<0.01$). In dynamic incubation of anterior pituitary, a 5-min pulse-chase with 10^{-7} M

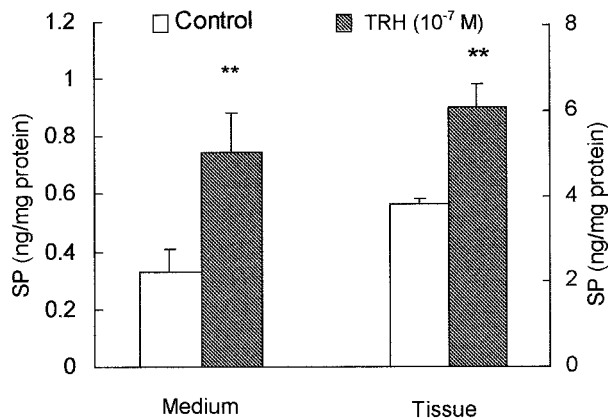


Figure 3 TRH stimulates ir-SP release and ir-SP content in anterior pituitary. Anterior pituitaries were incubated for 60 min in the presence of TRH or KRB (control). Bars represent means \pm S.E.M.S ($n=6$). Basal release of ir-SP was 0.200 ± 0.013 fmol/ml per anterior pituitary. Statistical differences between TRH and control were determined by Student's *t*-test. ** $P<0.01$.

TRH significantly increased ir-SP release vs control values. This increase persisted throughout the incubation period (Fig. 4). The concentration of ir-SP released in the last fraction was 28.50 ± 0.11 fmol/ml per anterior pituitary. SP released after TRH pulse-chase stimulus was approximately fourfold higher than that found in the 60-min period of uninterrupted static incubation with TRH. Prolactin release increased 15 min after the TRH pulse (1.623 ± 0.08 μ g/ml per anterior pituitary), then lowered to basal values (Fig. 4).

Effect of prolactin on ir-SP release

In order to evaluate the influence of prolactin on the effect of TRH on SP release, anterior pituitaries were incubated with VIP, another prolactin-releasing factor. VIP increased prolactin release (control: 0.941 ± 0.028 μ g/mg protein; 10^{-7} M VIP: 1.539 ± 0.121 , $P<0.01$), but did not affect ir-SP release (control: 0.776 ± 0.031 ng/mg protein; 10^{-7} M VIP: 0.718 ± 0.034 ; $n=5$). On the other hand, anti-prolactin serum increased ir-SP release and content. TRH and the anti-prolactin serum together had no additive effect on ir-SP release (Fig. 5).

Effect of simultaneous presence of TRH and SP on prolactin release

In order to study the interaction between TRH and SP in prolactin release, anterior pituitaries were incubated with either TRH or SP alone or both peptides together. Prolactin release was stimulated by increasing TRH concentrations (control: 1.022 ± 0.040 μ g/mg protein; 10^{-9} M TRH: 1.209 ± 0.042 ; 10^{-8} M TRH: 1.352 ± 0.037 *; 10^{-7} M TRH: 1.489 ± 0.100 **); 10^{-6} M TRH:

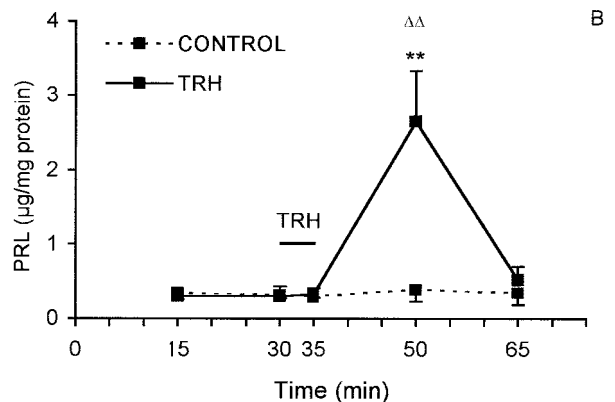
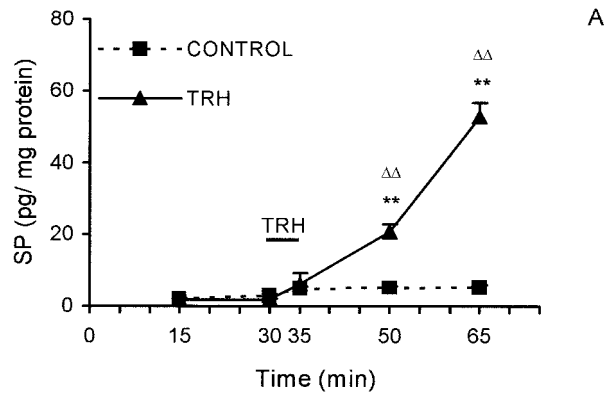


Figure 4 TRH stimulates ir-SP release and prolactin from anterior pituitary. Release of SP from anterior pituitary was evaluated by dynamic incubations as described in Materials and Methods. After 30 min of incubation (basal release), 10^{-7} M TRH was added for 5 min. Controls were run in parallel with KRB only. Values represent means \pm S.E.M.S of ir-SP (A) and prolactin (B) released in each fraction of the incubation period indicated ($n=6$). Basal release of ir-SP was 1.75 ± 0.06 fmol/ml per anterior pituitary. Statistical differences between basal and post-pulse release (** $P<0.01$) were determined by Dunnett's test. Statistical differences between the TRH and control groups for each time period ($\Delta\Delta P<0.01$) were determined by Student's *t*-test.

1.642 ± 0.069 ***, $n=7$, * $P<0.05$, ** $P<0.01$ vs control, Dunnett's test). When anterior pituitaries were incubated simultaneously with 10^{-7} M TRH and 10^{-7} M SP, the stimulatory effect of the two peptides together on prolactin release was similar to that observed for each alone (Fig. 6). SP at a concentration of 10^{-6} M also stimulated prolactin release but significantly reduced TRH-induced prolactin release, while 10^{-5} M SP, which failed to affect prolactin release, markedly decreased TRH stimulus.

Discussion

This study shows that TRH enhances ir-SP release from the anterior pituitary and that intrapituitary SP participates

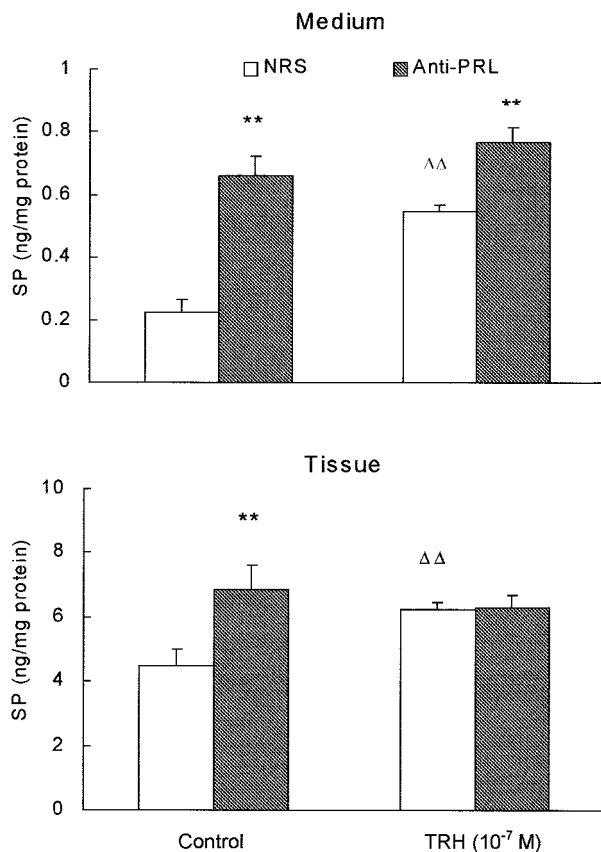


Figure 5 Effect of anti-prolactin serum on TRH-stimulated ir-SP release and content. Anterior pituitaries were incubated for 60 min with 10^{-7} M TRH and anti-prolactin serum (Anti-PRL, final dilution 1:200). Controls were incubated with normal rabbit serum (NRS, final dilution 1:200). Bars represent means \pm S.E.M.s ($n=5$). Basal release of ir-SP was 0.183 ± 0.033 fmol/ml per anterior pituitary. Statistical significance was determined by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test. ** $P < 0.01$ vs respective control without Anti-PRL. $\Delta\Delta P < 0.01$ vs respective control without TRH.

in TRH-induced prolactin release. TRH increased both prolactin and SP release. Said increases induced by TRH were higher when TRH was tested as a pulse (for 5 min) than when it was present for 60 min (static incubation). Rapid desensitization is characteristic of most native Gq/11-coupled receptors, like those for TRH or SP, in the permanent presence of an agonist (Sanders & LeVine 1996, Bhom *et al.* 1997, Yu & Hinkle 1998). Therefore, it is possible that continuous stimulation with TRH may desensitize the receptor. The effect of TRH on ir-SP does not seem to be a consequence of increased prolactin concentration since an increase of prolactin release induced by VIP did not affect ir-SP release. Also, an anti-prolactin serum increased basal ir-SP release, suggesting that the reduction of extracellular prolactin may increase ir-SP release. Similarly, others reported that chronic treatment

with a dopamine agonist, which decreases prolactin release, increases SP mRNA but does not modify ir-SP content in the rat anterior pituitary, suggesting that prolactin may decrease the synthesis and release of SP (O'Halloran *et al.* 1991). Ovariectomy, which also decreases prolactin release, raises SP content (De Palatis *et al.* 1985), whereas oestrogen administration increases prolactin release and decreases anterior pituitary SP (O'Halloran *et al.* 1990). Therefore, our results suggest that TRH-induced SP release would not be mediated by prolactin.

Besides, both TRH and anti-prolactin serum increased ir-SP content in the anterior pituitary. This enhancement of SP content could result from processing of the prepro-tachykinin protein and/or translation of the stored prepro-tachykinin mRNA, which encodes for SP and NKA (Farrow 1993, Weintraub 1995).

We previously observed that a specific anti-SP serum reduces *in vitro* basal prolactin release (Afione *et al.* 1990a) and markedly decreases hyperprolactinemia induced by anterior pituitary grafting under the kidney capsule (Debeljuk *et al.* 1988). In the present study, the anti-SP serum blocked TRH-induced prolactin release. The specific NK1 receptor antagonist, WIN 62,577, also inhibited TRH-induced prolactin release, supporting our results for the anti-serum. The present results confirm previous reports suggesting that intrapituitary SP participates in the regulation of basal prolactin release and show that SP is involved in the effect of TRH on prolactin release from anterior pituitary of normal male rats. Reports by Balsa *et al.* (1996) demonstrated that intrapituitary VIP mediates TRH-induced prolactin release from anterior pituitary cells, this effect being partial and dependent on thyroid hormones.

The present study also found interaction between TRH and SP that affected prolactin release. Both peptides together in equimolar concentration increased prolactin release, although no additive effect was observed, thus supporting other reports indicating that TRH and SP could share an effector mechanism (Mau *et al.* 1990). However, when the concentration of SP was higher than that of TRH in the incubation medium, a decrease in the stimulatory effect of TRH on prolactin release was observed. SP was reported to interact not only with its own specific receptor, but also with others, including some outside its own receptor family (Simasko *et al.* 1987, Shangochian & Leeman 1992, Goettl & Larson 1994, Maggi 1995). Sharif (1990) demonstrated that SP specifically reduced, in a concentration-dependent manner, TRH receptor binding in different areas of the central nervous system and in the pituitary gland. Although SP seems to compete with the TRH receptor, TRH does not compete with SP binding (Wormald *et al.* 1989). These facts and our results lead us to speculate that SP could interfere with TRH binding. Alternatively, it has been suggested that SP could affect post-receptor events, such

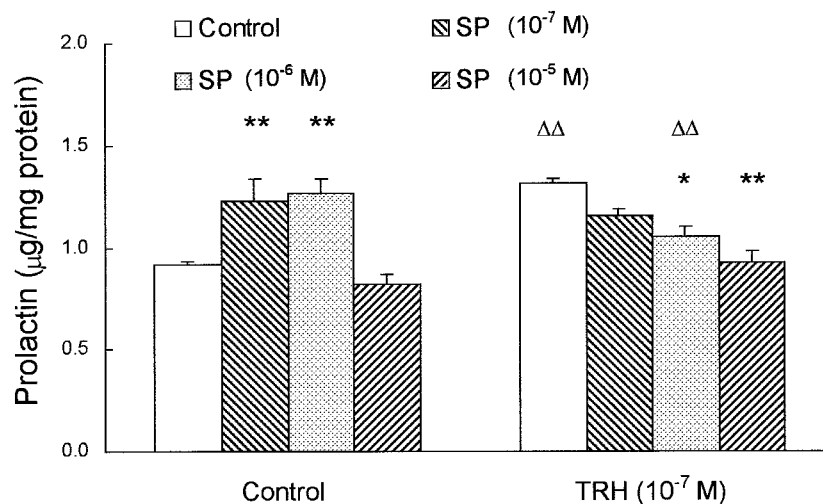


Figure 6 Effect of SP on TRH-induced prolactin release. Anterior pituitaries were incubated for 60 min with 10^{-7} M TRH and increasing concentrations of SP. Bars represent means \pm S.E.M.S ($n=6-7$). Statistical significance was evaluated by two-way ANOVA followed by Student–Newman–Keuls *post hoc* test. * $P<0.05$; ** $P<0.01$ vs respective control without TRH. $\Delta\Delta P<0.01$ vs respective control without SP.

as down-regulation of protein kinase C by high SP concentration (Ozawa *et al.* 1993).

Whatever the mechanism by which intrapituitary SP interacts with TRH in the control of prolactin release, SP may be relevant to the modulation of TRH action on lactotroph activity without modifying its effect on thyrotroph function (Arisawa *et al.* 1989). SP at low concentrations, such as those found in the environment of the target cell, may mediate TRH effect on prolactin release, while higher concentrations of SP may interfere with TRH effect on lactotrophs. Similarly, Shamgochian & Leeman (1992) reported that the same concentration of SP that stimulates LH release (10^{-6} M) also displaces gonadotropin-releasing hormone (GnRH) from anterior pituitary membranes. Also, SP inhibits GnRH-stimulated LH secretion from cultured pituitary cells (Wormald *et al.* 1989), suggesting that SP may act by preventing total exhaustion of the response capabilities of anterior pituitary cells.

Primary hypothyroidism shows sex-dependent changes in serum prolactin levels. Although hyperprolactinemia has been observed in female hypothyroid rats (Tohei *et al.* 1988), hypoprolactinemia is more frequent in both female and male hypothyroid rats (Sowers & Sollars 1980, Lloyd *et al.* 1990, Mizukami *et al.* 1993, Kimura & Furudate 1996, Tohei *et al.* 1997, Yamahnouchi *et al.* 1997). In these conditions a decrease in pituitary prolactin synthesis was observed (Vale *et al.* 1973, Seo *et al.* 1979, Jahnke *et al.* 1980), as well as an increase in SP and TSH synthesis (Arita *et al.* 1993, Jones *et al.* 1994) and in the cell population that secretes them (Lloyd *et al.* 1990). Therefore, the increase in intrapituitary SP induced by primary

hypothyroidism might interfere with the effect of TRH on lactotrophs without affecting TSH secretion.

In brief, the present study shows that TRH stimulates ir-SP release and increases ir-SP content in the anterior pituitary. In addition, a paracrine interaction between TRH and intrapituitary SP seems to control prolactin release. SP appears to have concentration-dependent, differential effects on prolactin release. SP may act as a physiological modulator of TRH effect on prolactin release and may also fine-tune the lactotroph response to TRH.

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