Dynamics of Prolactin Secretion from Diethylstilbestrol-Induced Rat Prolactinoma Tissue *In Vitro* (43934)

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Abstract. **Experiments were performed to determine whether PRL secretion in the rat diethylstilbestrol (DES)-induced prolactinoma model is affected by the addition of thyrotropin-releasing hormone (TRH) and/or immunoneutralization of intrapituitary vasoactive intestinal polypeptide (VIP) *in vitro*. Male Fischer 344 rats were implanted with either a 10 mg DES or placebo pellet 30 days prior to obtaining the anterior pituitary glands for perifusion. The anterior pituitaries were quartered and used in three different perifusion experiments. In Experiment I, placebo-treated tissue channels were perifused for 2 baseline hr followed consecutively by a 30-min exposure to 1:100 nonimmune rabbit serum (NRS), a 30-min wash, and a final 30-min exposure to 10^{-5} *M* TRH. Additional placebo channels were run as above except 1:100 VIP antiserum (AVIP) was substituted for NRS and AVIP was added to the TRH. In Experiment II, the same perifusion protocol was used as in Experiment I, except DES-induced tumor tissue was used instead of placebo tissue. Results from Experiment I and II reveal that AVIP significantly decreased PRL secretory rate in both DES and placebo groups. In the tumor group, both TRH alone and in the presence of AVIP significantly increased the PRL secretory rate. In Experiment III DES-induced tumor tissue channels were perifused with a similar protocol, except the concentrations of NRS and AVIP were increased to 1:10. Both NRS and AVIP significantly decreased PRL secretory rate; however, AVIP had a significantly greater effect than NRS. In this experiment, 1:10 AVIP overcame the stimulatory effect of TRH.**

In conclusion, AVIP decreases and TRH increases, even in the presence of AVIP, PRL release in DES-induced prolactinoma tissue *in vitro*. Increasing the AVIP concentration 10-fold diminished the PRL-releasing action of TRH in the tumor tissue. These data suggest that PRL secretion is not autonomous in these prolactinomas and can be affected by exogenous TRH and partial immunoneutralization of endogenous VIP. [P.S.E.B.M. 1995, Vol 210]

Vasoactive intestinal polypeptide (VIP), a 28 amino acid peptide, was first identified in porcine duodenum (1). Further investigation has demonstrated this peptide to be a widely distributed neuromodulator found in numerous areas of the central nervous system (2–4). VIP is found in high concentrations in the hypothalamus (5, 6) and hypophyseal blood (7), and stimulates prolactin (PRL) release *in vivo* (8–10) and *in vitro* (11–15), demonstrating that this peptide is a PRL-releasing factor. In addition, this laboratory (16) and others (17, 18) have previously reported intrapituitary VIP affects basal PRL release and is synthesized in anterior pituitary tissue (19). Two studies have suggested two different roles for intrapituitary VIP: one as an autocrine regulator of PRL secretion using a reverse hemolytic plaque assay (17) and the other as a paracrine regulator of PRL secretion where immunostainable VIP was found in stellate, neuron-like cells in pituitary tissue of hypothyroid rats (18). The exact role of VIP in regulation of PRL se-

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cretion is unclear. However, this laboratory has recently shown that both VIP and thyrotropin-releasing hormone (TRH) affect the newly synthesized PRL pool and not the stored PRL pool in normal rat anterior pituitary tissue (20).

The administration of high levels of estrogen over time in certain strains of rats is a well-established method of inducing prolactin-secreting tumors of the anterior pituitary gland (21–27). The mechanism for induction of these tumors by estrogen is not clearly understood. Possibilities include a direct stimulatory effect on the pituitary lactotrope, modulation of hypothalamic inhibitory or stimulatory factors, and alteration of pituitary responsiveness to prolactin-regulating factors, or a combination of these mechanisms. Additionally, the regulatory capacity versus autonomy of these tumors has not been characterized. This study was designed to test the influence of two PRL-releasing factors, TRH and VIP, on PRL secretion in both normal anterior pituitary and diethylstilbestrol-induced prolactinoma tissue.

Materials and Methods

Pituitary Tissue. Male Fischer 344 rats (Sasco/King Animal Laboratories, Oregon, WI) weighing 150–175 g (age 55–65 days) were used for all experiments. Animals were housed at 23°C with a 12:12-hr light:dark cycle, with lights on at 0600. Laboratory animal chow and tap water were provided *ad libitum*. Diethylstilbestrol (DES) and placebo pellets (Innovative Research of America, Toledo, OH) were implanted subcutaneously between the scapulae under anesthesia. Each DES pellet contained 10 mg of DES. Subcutaneous implantation of this form of DES pellet results in approximately three weeks of controlled product release per manufacturer's specification. Subcutaneous implantation of estrogens has been shown previously to produce PRL-secreting pituitary tumors in the Fischer 344 strain of rats (23, 26). The anesthetic used was ketamine HCl injected im at a dose of 10 mg/100 g body wt. Acepromazine was added as an adjuvant to the ketamine HCl at a dose of 1.1 ml/10 ml ketamine.

After 30 days, animals were brought to a separate procedure room and sacrificed by rapid decapitation to minimize stress-induced PRL stimulation. Pituitary glands were removed with ophthalmic forceps and the posterior lobe was gently separated and discarded. Anterior pituitary lobes were placed on a filter paper moistened with oxygenated minimal essential medium (MEM) (Gibco Laboratories, Grand Island, NY) and transported to the laboratory. Trunk blood was collected from the rats used in Experiment I and II.

Pituitary Perifusion. Anterior pituitaries were weighed, quartered, and perifused, 12 quarters per channel, for a total of 3.5 hr, at a rate of 200 μl/min in Medium 199 (M199) containing 1% bovine serum albumin, 1% nonessential amino acids, 1 m*M* glutamine, and 1% Pen/Strep. Medium was equilibrated with 95% O_2 and 5% CO_2 during perifusion. Perifusions were performed with the pituitary quarters supported on a 5-μm pore-type SM Millipore filter contained in a 25-mm Swinnex filter holder (Millipore Corp., Bedford, MA). Fractions of 1 ml were obtained at 5-min intervals. The amount of PRL in each fraction was measured using radioimmunoassay (RIA). In the experiments described below, nonimmune rabbit serum (NRS) and VIP antiserum (AVIP) were utilized during perifusion. These sera were partially purified, on a Sephadex G-75 column, as previously described (16).

Experiment I. Two placebo channels were perifused for 2 baseline hr in M199 followed consecutively by a 30-min exposure to 1:100 NRS, a 30-min wash period, and a final 30-min exposure to 10^{-5} *M* TRH. An additional two placebo channels were run as above, except 1:100 AVIP was substituted for NRS and 1:100 AVIP was added to the TRH. This experiment was repeated to give a total *n* of 4 for each placebo protocol. The perifusion protocols for Experiment I are illustrated in Figure 1. Serum PRL, VIP, growth hormone (GH), and testosterone concentrations were determined in trunk blood by RIA.

Experiment II. Two DES channels were perifused for 2 baseline hr in M199 followed consecutively by a 30-min exposure to 1:100 NRS, a 30-min wash period, and a final 30-min exposure to 10^{-5} *M* TRH. An additional two DES channels were run as above except 1:100 AVIP was substituted for 1:100 NRS and 1:100 AVIP was added to the TRH. This experiment was repeated to give a total *n* of 6 for each DES protocol. The perifusion protocols for Experiment II are illustrated in Figure 2. Serum PRL, VIP, GH, and testosterone concentrations were determined in trunk blood by RIA.

Experiment III. Three DES channels were perifused for 2 baseline hr in M199 followed consecutively by a 30-min exposure to 1:10 NRS, a 30-min wash period, and a final 30-min exposure to 1:10 NRS plus 10^{-5} *M* TRH. An additional three DES channels were run as above, except 1:10 AVIP was substituted for 1:10 NRS. The perifusion protocols for Experiment III are illustrated in Figure 3.

Radioimmunoassays. The rPRL and rGH radioimmunoassays (RIAs) were performed with materials generously provided by the NIDDK/National Hormone and Pituitary Programs, and for these experiments reference preparation RP-3 and RP-2 were used, respectively. In our laboratory, the intraassay and interassay coefficients of variation are 2.9% and 12.7%, respectively, for the rPRL assay. Intra- and interassay

coefficients of variation for rGH are 2.7% and 7.8%, respectively. Testosterone was measured using an RIA kit (Diagnostics Products Corp., Los Angeles, CA) with intra- and interassay coefficients of variation being 5.0% and 10.8%, respectively. The rVIP RIA was performed using a previously described method (19) with intra- and interassay coefficient of variation being 5.0% and 10.0%, respectively. The rPRL radioimmunoassay uses rabbit anti-PRL, and a final concentration of 1:300 NRS as a carrier. Nonetheless, in view of the fact that some experiments would include 1:10 NRS or AVIP in the samples, we assessed the potential effect of this on the assay. Inclusion of appropriately diluted aliquots of perifusion medium containing NRS resulting in conditions identical to the above mentioned experiments had no effect on binding of radiolabeled PRL (data not shown).

Data Analysis. Descriptive statistics and independent Student's *t* test were used to compare the DES-induced versus placebo effects in the male Fischer rats as displayed in Table I. Significance was determined at $P < 0.05$.

Data obtained during the perifusion periods was depicted as the rate of release of PRL into the perifusion media and expressed as nanograms per milliliter per minute. For each phase of the perifusion period, the mean value of the last four fractions collected was used for comparison purposes. These fractions were used to ensure that the pituitary quarters were adequately exposed to the new test condition, in order to account for the initial dead space (10 min or 2 ml) in the perifusion apparatus. Additionally, these last four fractions included the biological or peak effects of the test condition and adjusted for tissue hormone releasing variability.

Data in Experiment I, II, and III were analyzed for normality and variance homogeneity. For these experiments, *n* is equal to the number of channels for a given perifusion protocol. Repeated measures analysis of variance (ANOVA) with post hoc hypothesis testing was utilized when no *a priori* hypothesis was entertained. In specific instances where an *a priori* hypothesis existed, independent *t* test comparing between perifusion protocols and paired *t* test comparing within perifusion protocols were utilized. The bars in the graphs represent standard error of the mean. Significance was determined at $P < 0.05$.

Results

The mean total body weight, mean anterior pituitary weight, and trunk blood PRL, VIP, GH, and testosterone values are shown in Table I for both DES- and placebo-treated rats. Basal PRL secretory rate determined during the initial 2 hr of perifusion was 10-fold higher in the DES channels than in the control channels, 282.2 ± 22.7 ng/ml/min vs 23.2 ± 2.0 ng/ml/min, respectively ($P < 0.001$).

Experiment I. All data for this experiment are depicted in Figure 1. Only AVIP at a 1:100 dilution significantly decreased the amount of PRL released into the perifusate in comparison with the corresponding and preceding basal perifusion period. NRS at this dilution had no effect.

When comparing the effects of a 30-min exposure to 10^{-5} *M* TRH alone on PRL secretion in placebo anterior pituitary tissue, TRH did not affect the amount of PRL released into the perifusate compared with the preceding wash period. Furthermore, with the addition of TRH, the 1:100 AVIP still significantly decreased the amount of PRL released compared with the preceding wash period in this group.

Experiment II. All data for Exp. II are depicted in Figure 2. As was seen in the placebo group, 1:100 AVIP significantly decreased the amount of PRL released into the perifusate compared with the preceding baseline in the DES tumor group. Again, NRS had no effect. In contrast to the placebo group, 10^{-5} *M* TRH, even in the presence of 1:100 AVIP, significantly increased the amount of PRL released into the perifusate compared with the preceding wash period.

Experiment III. All data for this experiment are depicted in Figure 3. Both NRS and AVIP at the 1:10 concentration significantly decreased the amount of PRL released into the perifusate when compared with their corresponding baseline values. However, when comparing the effects of AVIP and NRS with each other, AVIP caused a significantly greater decrease in PRL release than NRS (Fig. 3). In contrast to Experiment II, 10^{-5} *M* TRH in the presence of 1:10 AVIP

Table I. Comparison of the Mean Total Body and Anterior Pituitary Weights and Trunk Blood PRL, VIP, GH, and Testosterone Values between DES- and Placebo-Treated Rats

Treatment	Total body weight (g)	Ant. pit. weight (mg)	Serum PRL (ng/ml)	Serum VIP (ng/ml)	Serum GH (ng/ml)	Serum testosterone (ng/ml)
DES (n = 12)	176.0 ± 3.9	14.6 ± 0.8	238.7 ± 25.8	1.2 ± 0.3	32.7 ± 3.2	0.1 ± 0.1
Placebo (n = 8)	261.5 ± 3.3	6.0 ± 0.5	73.0 ± 14.9	8.1 ± 2.3	27.9 ± 1.7	1.9 ± 0.4
	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.008$	NS	$P < 0.001$

Note. Values are expressed as the mean ± SEM. Ant. pit., anterior pituitary; NS, not significant.

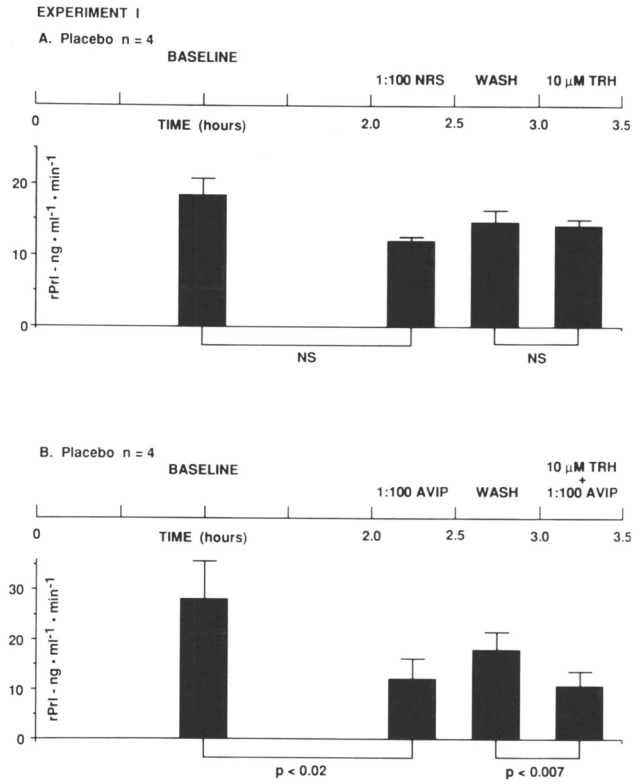

Figure 1. Effect of AVIP and TRH ± AVIP on PRL secretory rate on placebo tissue, *in vitro*. The two perifusion protocols used in Experiment I using anterior pituitary quarters from placebo-treated F344 rats are illustrated. The mean PRL secretory rate ± SEM of the last four fractions collected is illustrated by the bar graphs located directly below each different test condition. *n*, the total number of channels used for each perifusion protocol.

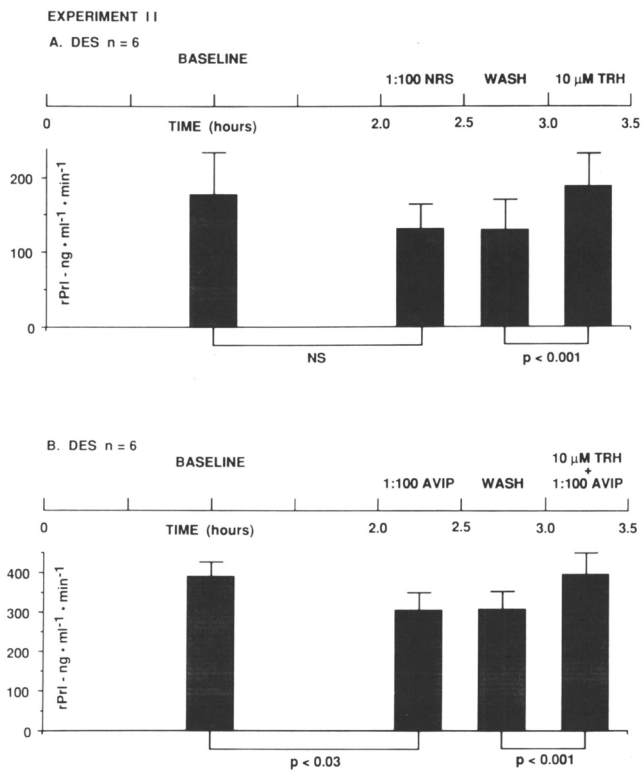

Figure 2. Effect of AVIP and TRH ± AVIP on PRL secretory rate on DES-induced prolactinoma tissue, *in vitro*. The two perifusion protocols used in Experiment II using anterior pituitary quarters from DES-treated F344 rats are illustrated. The mean PRL secretory rate ± SEM of the last four fractions collected is illustrated by the bar graphs located directly below each different test condition. *n*, the total number of channels used for each perifusion protocol.

did not increase the amount of PRL released into the media.

Discussion

This study was designed to determine whether PRL secretion in rat DES-induced prolactinomas is autonomous or regulable by known PRL secretogogues. The answer to this question would allow the use of this rat model for gaining a better understanding of the pathogenesis and pathophysiology of estrogen-induced prolactinomas. The effects of AVIP and TRH, a known inhibitor and stimulant of PRL secretion, respectively, were studied using an *in vitro* perifusion system.

Male rats were used to preclude the need for oophorectomy. Although the DES pellets result in 3 weeks of controlled product release, per the manufacturers specifications, rats were sacrificed after 30 days to ensure each rat received the full 10-mg dose of DES, and to assure that the DES had fully cleared. Thus, the effects of DES on the experimental group can be assumed to be secondary to permanent changes in the pituitary, not activational effects of DES. In order to confirm prolactinoma formation, comparisons of anterior pituitary weight, serum PRL levels and basal PRL secretory rate were made between the DES- and placebo-treated rats. Trunk blood and basal perifusate prolactin concentrations increased significantly as expected after the 30-day exposure to high doses of DES implanted in male Fischer 344 rats. This stimulation of hormone production and secretion has been shown by Phelps *et al.* (26) through immunocytochemical PRL staining, separation on Ficoll-Hypaque, and unit gravity sedimentation to accompany a significant increase in rat anterior pituitary weight through lactotrope hyperplasia and hypertrophy, and a significant increase in the prolactin content per cell. Similarly, our study revealed a significant increase in mean anterior pituitary weight in rats treated with DES. In contrast, the mean total body weight was significantly lower in the DES-treated animals than in controls. Because of this difference in body weight between the two groups, trunk blood GH and testosterone levels were measured. There was no difference in serum GH levels between the DES- and placebo-treated rats. However, testosterone levels were noted to be significantly lower in the DES group. While specific experiments were not performed to address the mechanism of this hypogonadism, three possible explanations seem likely: Inhibition of gonadotropin secretion by the high

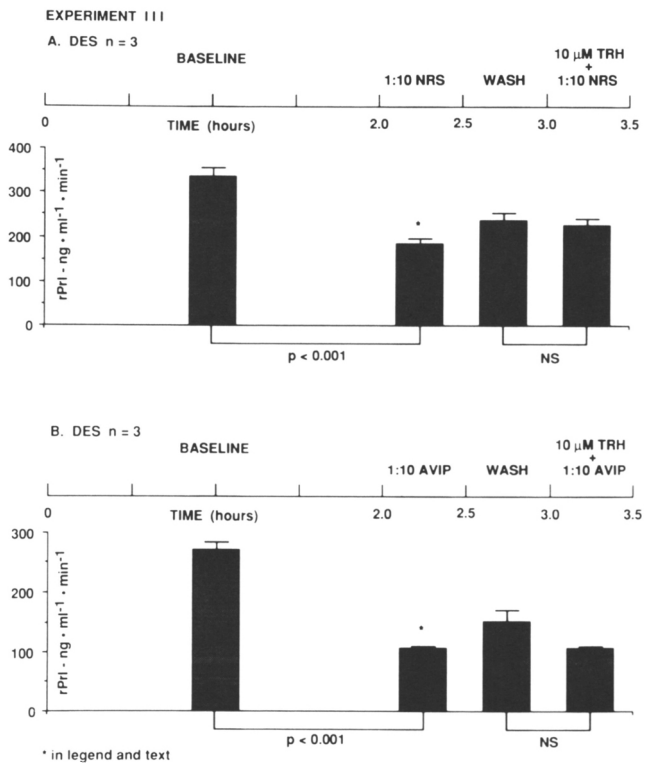

Figure 3. Effect of increasing the AVIP concentration on the PRL-releasing action of TRH in DES-induced prolactinoma tissue, *in vitro*. The two perifusion protocols used in Experiment III using anterior pituitary quarters from DES-treated F344 rats are illustrated. The mean PRL secretory rate ± SEM of the last four fractions collected is illustrated by the bar graphs located directly below each different test condition. *n*, the total number of channels used for each perifusion protocol. $^*P < 0.001$ between mean PRL secretory rate ± SEM of 1:10 NRS (Protocol A) and 1:10 AVIP (Protocol B).

estrogen levels, the high PRL levels, or direct obliteration of gonadotropes by the tumor.

While studies by others (28, 29) have revealed an increase in intrapituitary VIP content in Fischer rats treated with estradiol, trunk blood VIP concentration in our Fischer 344 rats treated with DES was significantly lower than in the placebo group. This decrease in serum VIP may be due to an inhibitory or negative feedback effect of high serum PRL levels on total body VIP. Prysor-Jones *et al.* (29) have shown this inhibitory effect of elevated plasma PRL on pituitary VIP content when the hyperprolactinemia is due to suppression of dopamine. This inhibition of pituitary VIP secondary to hyperprolactinemia was not seen in estrogen-treated rats (28, 29); on the contrary, an increase in the amount of intrapituitary VIP was noted under these conditions. In contrast, hypothalamic VIP content was noted to significantly decrease in rats treated with estrogens (28). The effect of hyperprolactinemia on trunk blood VIP concentration has not been investigated. These studies suggest hyperprolactinemia decreases VIP concentration at multiple sites, including trunk blood, hypothalamus, and anterior pituitary, except in the presence of estrogen where this inhibition is specifically overridden at the level of the anterior pituitary. This observation implies a direct or indirect stimulatory effect of estrogens on intrapituitary VIP.

As shown in Experiment I and II, only AVIP significantly decreased the amount of PRL released into the perifusate in both the placebo and DES groups. NRS at this same dilution of 1:100 did not significantly affect PRL release. In contrast, when the concentration was increased 10-fold, both NRS and AVIP significantly decreased PRL release in the DES group. This decrease in PRL implies a nonspecific protein inhibitory effect at the higher concentrations; however, the effect of AVIP was significantly greater than that of NRS.

TRH at a concentration of 10^{-5} *M* significantly stimulated PRL release in the DES group but failed to affect the placebo group, for which we have no explanation. In the presence of 1:100 AVIP, the stimulatory effect of TRH in the DES group remained significant. TRH failed to stimulate PRL release in the DES group when the concentration of AVIP was increased to 1:10. This TRH/AVIP response in the DES tumor model may be explained by three complementary mechanisms. First, estrogens have been found to increase the number of TRH receptor sites on the lactotrope (30). An increase in the number of TRH binding sites on the lactotrope would permit a greater PRL response at a lower concentration of TRH. In this case, the DES-induced prolactinoma model would be more sensitive to the known stimulatory effects of TRH than normal anterior pituitary tissue due to this increase number of TRH receptor sites. Second, estrogen has been shown to be toxic to tuberoinfundibular dopaminergic neurons (21, 25). Although the estrogen toxicity to these neurons results in a long-term decrease in dopamine, removal of the anterior pituitary for the perifusion experiments results in a rapid removal of remaining hypothalamic dopamine. Martinez de la Escalera and Weiner (31, 32) demonstrated the removal of dopamine not only results directly in the release of PRL, but the "brief" removal of dopamine results in the long-term potentiation of the PRL-releasing action of TRH. This potentiation of TRH action on PRL secretion due to dopamine withdrawal appears to be mediated by the action of protein kinase A since pretreatment with VIP, a hormone that signals via protein kinase A, also potentiates the action of TRH (32). The increase in intrapituitary VIP content seen in the estrogen-induced prolactinomas could have its own direct effect on increasing PRL release and prolactinoma formation and also, have an indirect effect through its potentiation of the PRL-releasing action of TRH. Third, estrogens have been reported to increase the amount of intrapituitary VIP (28, 29). If

estrogen increases the amount of intrapituitary VIP, it would take more VIP antiserum to neutralize this peptide in the DES-induced prolactinoma model. This laboratory has observed this increase in pituitary VIP content when the same number of DES-induced tumor cells as normal anterior pituitary cells required 10-fold higher concentrations of AVIP to significantly decrease the amount of PRL released into the medium (data not shown).

In conclusion, these data demonstrate that PRL secretion is not autonomous in the DES-induced prolactinoma model and is indeed regulated by TRH. In addition, immunoneutralization of VIP significantly inhibits PRL secretion in this model as originally reported by this laboratory in normal rat anterior pituitary tissue (16). The increased sensitivity of the PR-Loma tissue to TRH may be due to changes caused by the estrogen, unique to the pituitary, resulting in potentiation of the PRL-releasing action of TRH.

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