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Estrogen attenuates expression of calcitonin-like immunoreactivity in the anterior pituitary gland

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Estrogens increase prolactin (PRL) synthesis and release in rats and humans, whereas pituitary-derived calcitonin-like immunoreactive peptide (pit-CT) inhibits PRL gene expression and release. To test the hypothesis that estrogens stimulate lactotrophs by diminishing pit-CT expression, the present studies examined effects of ovariectomy (ovx) and estradiol (E2) administration on (1) pit-CT IR cell population; (2) pit-CT IR content and (3) release of pit-CT IR by cultured anterior pituitary (AP) cells. Ability of anti-calcitonin immunoglobulins (anti-CT IgG) to stimulate PRL release from cultured AP cells was also examined. The results suggest that ovariectomy induced a large increase in pit-CT IR cell population in the AP gland and E2-treatment dramatically reversed this increase. Similar changes were observed in pit-CT IR content of AP extracts. Cultured AP cells from ovx rats released significantly higher amounts of pit-CT IR, and anti-CT IgG induced a significant increase in basal PRL release. AP cells from E2-treated rats secreted lower amounts of pit-CT IR and this was associated with significantly higher PRL release. These results suggest that estrogens may stimulate lactotrophs, at least in part, by removing inhibitory influence of endogenous pit-CT.

Keywords: estrogen; calcitonen; inhibition; prolactin; pituitary

Introduction

Calcitonin is a 32-amino acid peptide secreted by the thyroid gland. In addition to the thyroid gland, calcitonin (CT) or related peptide(s) are widely expressed in a number of tissues including the anterior pituitary (AP) gland (Potts *et al.*, 1976). Recent findings from this and other laboratories have shown that exogenously added CT selectively and potently inhibits basal and TRH-stimulated prolactin gene expression and release from rat AP cells (Shah *et al.*, 1988; Judd *et al.*, 1990). CT does not affect basal GH, TSH, FSH and LH release as well as TRH-induced TSH or GnRH-induced LH release (Shah *et al.*, 1990). These studies have further shown that immunoreactive CT-like peptide is synthesized and released from cultured rat AP cells and rabbit anti-CT serum induces a significant increase in prolactin release from cultured rat AP cells (Shah *et al.*, 1993). Although the precise sequence of pituitary CT-like immunoreactive peptide (pit-CT IR) has not been determined, it seems to share antigenic sites with salmon (s) and human (h) CT peptides. Anti-sCT and hCT-IgG immunoprecipitate similar molecular species from AP cell lysates, anti-sCT IgG labels a specific cell population of the AP gland, and the additions of these IgGs to AP cell cultures induce a significant increase in PRL release (Shah *et al.*, 1993). These results suggest that pit-CT-IR originates from the AP gland and serves as a paracrine inhibitor of prolactin release.

In contrast to the inhibitory actions of pit-CT, estradiol is known to increase serum PRL levels in man and rats (Neill, 1988). Stimulatory actions of estrogens on lactotrophs have been shown to occur through multiple mechanisms. For example, estrogens have been shown to stimulate PRL gene transcription (Maurer, 1982). In addition, estrogens down-regulate dopamine receptors, decrease hypothalamic dopamine synthesis and increase TRH receptors in the AP gland (Giguere *et al.*, 1982; Pilotte *et al.*, 1984). Recent evidence suggests that estrogens also modulate the expression of various paracrine/autocrine factors including vaso active intestinal peptide (VIP) and galanin (Kaplan *et al.*, 1988; Lam *et al.*, 1990). Expression of VIP as well as galanin is induced by estrogens and these peptides have been shown to stimulate PRL release and induce lactotroph proliferation through paracrine/autocrine actions (Prysor-Jones *et al.*, 1989; Wynik *et al.*, 1993). Since pit-CT may serve as a paracrine inhibitor of PRL release, an important objective of the present investigations was to examine whether estrogens modulate pit-CT expression in the AP gland. Effects of estrogen status on pit-CT expression were examined by (1) investigating the changes in pit-CT immunopositive cell populations of the AP gland following ovariectomy (ovx) and E2 replacement; (2) assessing pit-CT content and release from AP glands after ovx and E2 replacement; and (3) by comparing anti-sCT IgG-induced PRL release from AP cell cultures of ovx and E2-treated rats.

Results

Hormonal manipulations and serum PRL levels

Ovariectomy-induced changes in pit-CT IR expression were examined ten days after the surgery because this duration of ovarian hormones deprivation produces significant changes in the expression of PRL as well as other pituitary paracrine peptides (Lam *et al.*, 1990; O'Halloran *et al.*, 1990). E2-replacement regimen was 3 days of silastic implantation as previously characterized (Pilotte *et al.*, 1984). This treatment caused a four to sixfold increase in serum PRL levels of ovx rats (Table 1).

pit-CT IR in the AP gland: immunocytochemistry

Initial experiments examined specificity of pit-CT IR staining in three concurrent sets of controls. In the first set of controls, anti-sCT IgG was replaced with non-immune IgG. These controls did not exhibit any staining (Figure C1). In a second set, anti-sCT IgG was preabsorbed with 1 μM of synthetic sCT solution for 1 h at 37°C. This pre incubation completely abolished pit-CT IR staining in AP tissue sections (Figure C2). In a third set of controls, cross-reactivity of anti-sCT IgG with CGRP-α and CGRP-β was tested. Anti-

Table 1 Effect of estrogen treatment on serum PRL levels

Hormone treatment	*Serum PRL levels (ng/ml) mean ± SEM*
Ovariectomized rats ($n = 6$)	6.6 ± 0.735
Estradiol-treated ovx rats ($n = 6$)	29.6 ± 7.6

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sCT IgG was preincubated with 1 μM of each of these peptides for 1 h at 37°C. These peptides did not affect pit-CT IR staining (Figure C3–C5).

In a next series of experiments, pit-CT IR distribution was examined in AP sections obtained from eugonadal, ovx and E2-replaced ovx rats (Figures 1A, B and C). The results suggest that AP sections from ovx rats contained relatively larger pit-CT IR cell populations, and they were distributed throughout the AP gland (Figures 1A and D). In contrast, pit-CT IR cells formed a much smaller cell population in eugonadal and E2-replaced AP glands (Figures 1B, C and E). Large differences in the expression of pit-CT IR between ovx and E2-replaced AP glands can be more clearly observed at a lower magnification (100 ×, Figures 1D and E). Average number of pit-CT IR cells in these sections was determined by counting the immunopositive cells from 20 fields at 200 ×

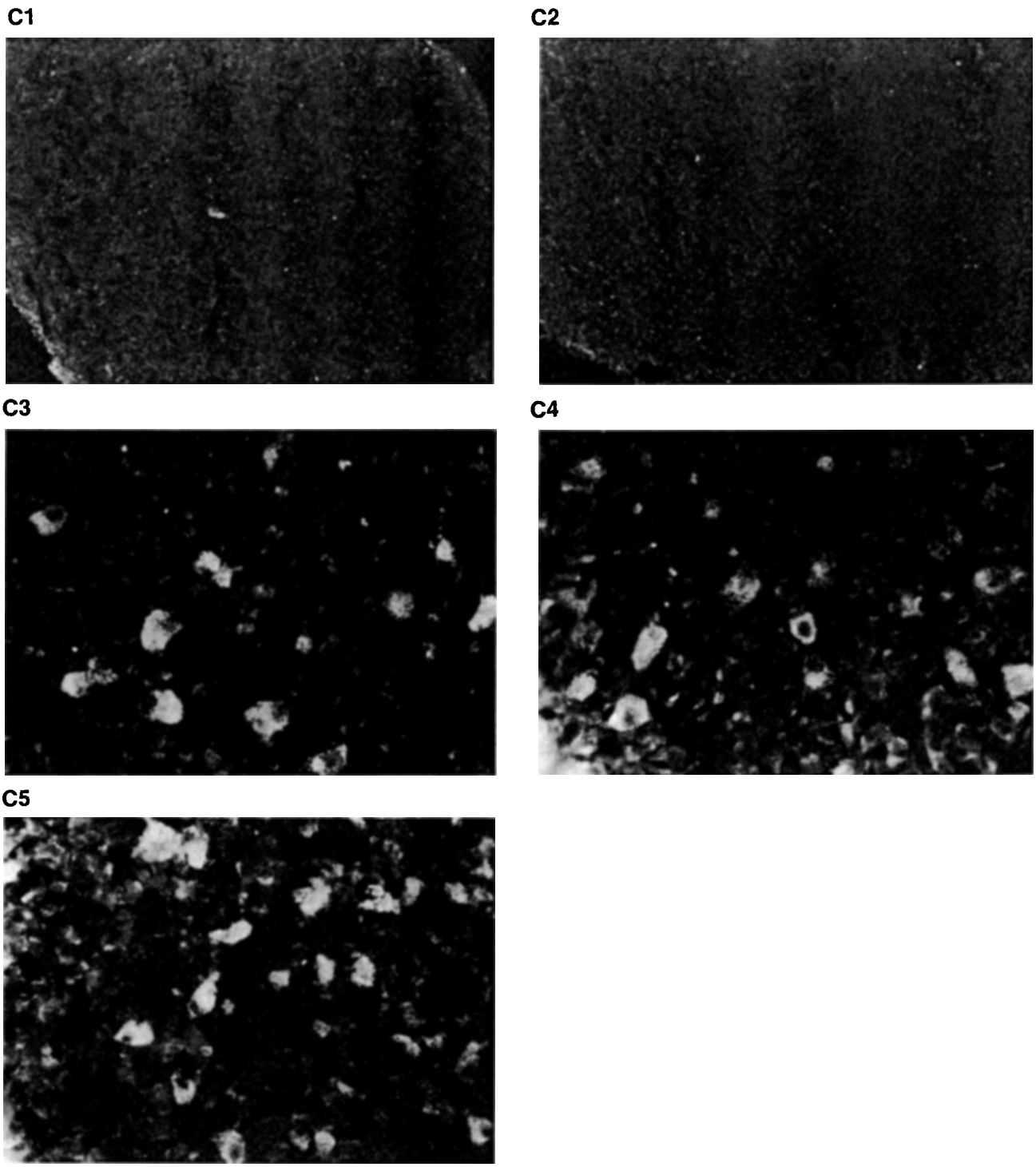

Figure C Specificity of pit-CT IR staining: controls. **C1:** A photomicrograph of a frozen AP section which was stained with non-immune IgG as described in the Methods sections. Magnification, 100 ×. **C2:** A photomicrograph of a frozen AP section. The section was stained with anti-sCT IgG that was preabsorbed with 1 μM sCT at 37°C for 1 h. No specific staining was observed, however, there was some increase in background staining. Magnification, 100 ×. **C3.** A photomicrograph of an AP section stained with anti-sCT IgG. Magnification, 400 ×. **C4.** A photograph of an AP section that was processed exactly as described in C3 but the anti-sCT IgG was preabsorbed with 1 μM CGRP-α as described in the Methods section. Magnification, 400 ×. **C5.** A photograph of an AP section that was processed exactly as described in C3 but the anti-sCT IgG was preabsorbed with 1 μM CGRP-β as described in the Methods section. Magnification, 400 ×.

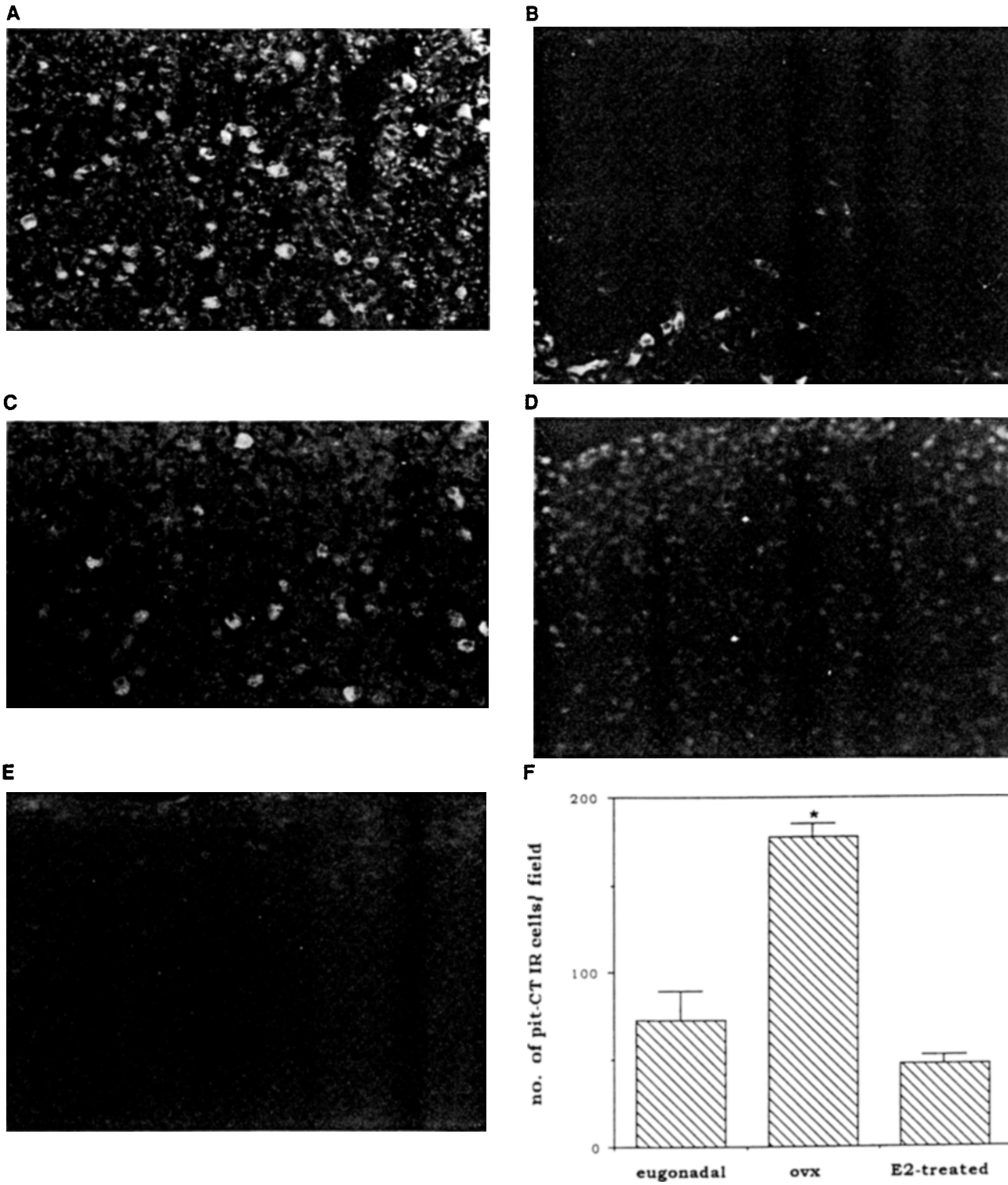

Figure 1 Effect of ovarian hormonal manipulations on Pit-CT IR cell populations in rat AP glands. (**A**) A typical fluoromicrograph of AP sections from ovx adult female rat. The AP gland was collected ten days after the surgery, and frozen sections (8–10 μm thick) were processed for pit-CT ICC as described in Methods section. These sections show numerous cells with significant pit-CT IR. Magnification, 200 ×. (**B**) A typical AP frozen section from gonadally-intact adult female rat depicting pit-CT IR cells. The APs were collected randomly (day of their estrus cycle was not examined). These sections show sparse distribution of pit-CT IR cells. Magnification, 200 ×. (**C**) A typical AP frozen section from E2-treated adult female rats.a The rats were ovx for 10 days, and were treated with estradiol silastic capsules for 3 days as described in the Methods section. Three days of E2-treatment causes a dramatic decline in pit-CT IR staining. Magnification, 200 ×. (**D**) Differences in pit-CT IR distribution can be more clearly observed at a lower magnification. A typical frozen AP section from ovx rats (10 days after the surgery). Numerous pit-CT IR cells are distributed throughout the section. Magnification, 100 ×. (**E**) A typical frozen section from E2-treated rat. The rats in this group were ovx for 10 days and implanted with estradiol capsules for 3 days. A dramatic decline in pit-CT IR cells is seen, and almost absence of pit-CT IR cells in the central region of the gland. Magnification, 100 ×. (**F**) Average distribution of pit-CT IR cells per field was determined at 200 ×. At least 20 fields from each specimen were counted, and three specimens from each treatment were examined. The data are presented as number of pit-CT IR cells per field (200 ×). Differences in pit-CT IR frequency were evaluated by one way ANOVA, and the level of significance was derived from Newman-keuls test. $^{*}P<0.001$ (difference between ovx *v* E2-treated or eugonadal)

magnification. Slides from at least three separate AP glands for each treatment group were examined. The results presented in Figure 1F show that pit-CT IR was detected in 72.4 ± 17.15 cells' per field in gonadally-intact female rats (on random days of the cycle). Ten days after ovariectomy, this number increased to 177 ± 8.05 pit-CR IR cells per field. Ovariectomized rats when treated with E2 capsules for 3 days, their pit-CT IR cell population quickly declined to 47.5 ± 4.93 per field.

In a second experiment, dispersed AP cells from ovx and E2-treated ovx rats were stained for pit-CT IR. It was observed that 7.02% ± 0.21 (mean ± SEM) cells stained for pit-CT IR from ovx rats. In contrast, only 2.1 ± 0.053% of cells stained for pit-CT from E2-treated ovx rats (Figure 2).

Effect of ovariectomy and estrogen treatment on pit-CT content of the AP gland

In a second set of experiments, effects of ovarian hormone manipulations pit-CT IR content was examined. The results presented in Figure 3A show that AP extracts and sCT exhibited parallelism in a sCT RIA. Hormonal manipulations caused large differences in pit-CT IR concentrations (Figure 3B). AP extracts of ovx rats contained almost threefold higher levels of pit-CT IR as compared to those from eugonadal or E2-treated rats.

Effect of ovarian hormone manipulations on pit-CT IR release: immunoreactivity and bioactivity

Since ovx and E2-treated rats exhibited large differences in pit-CT IR expression, next group of experiments tested whether these treatments alter secretion of pit-CT IR and/or its inhibitory influence on PRL secretion. This was examined by (a) studying pit-CT IR release by cultured AP cells, and (b) by testing the ability of anti-sCT IgG to stimulate PRL release from AP cells.

(a) pit-CT IR release AP cells from ovx and E2-treated rats were cultured and conditioned media was collected after 24 h. pit-CT IR and PRL levels in the media were analysed by RIAs as previously described. The results suggest that AP cells from ovx rats released sixfold higher amounts pit-CT IR in 24 h cultures as compared to those from E2-treated rats (Figure 4A). In contrast, AP cells from E2-treated rats released three-fold higher amounts of PRL as compared to those from ovx rats (Figure 4B). These results suggest a negative relationship between pit-CT IR and PRL release. Furthermore, results, that E2-treatment caused a large increase in PRL release, demonstrate the effectiveness of E2 treatment.

(2) bioactivity of pit-CT released Since pit-CT is a putative paracrine inhibitor of PRL release, its immunoneutralization should increase the PRL release. Thus, anti-sCT IgG-induced PRL release could provide an index for biological activity of endogenous pit-CT release. This group of experiments tested the effects of anti-sCT IgG on PRL release in AP cells obtained from ovx and E2-replaced rats. The results presented in Figure 5 suggest that anti-sCT IgG induced an increase in PRL release in AP cells from ovx as well as E2-replaced rats. However, the increase in PRL release from AP cells from ovx rats was 60% over non-immune IgG-treated cont-

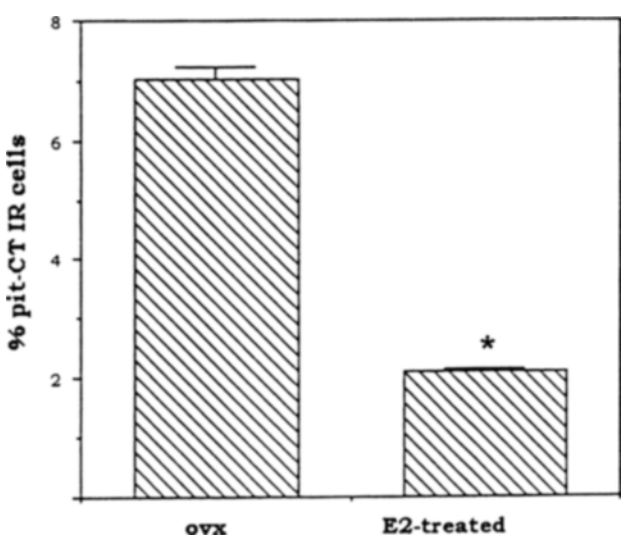

Figure 2 Percentage distribution of pit-CT IR cells in dispersed AP cells from ovx and E2-treated rats was determined as described in the Methods section. Differences in these cell populations were evaluated by *t*-test and significance was derived from a two-tailed table. $^*P<0.01$

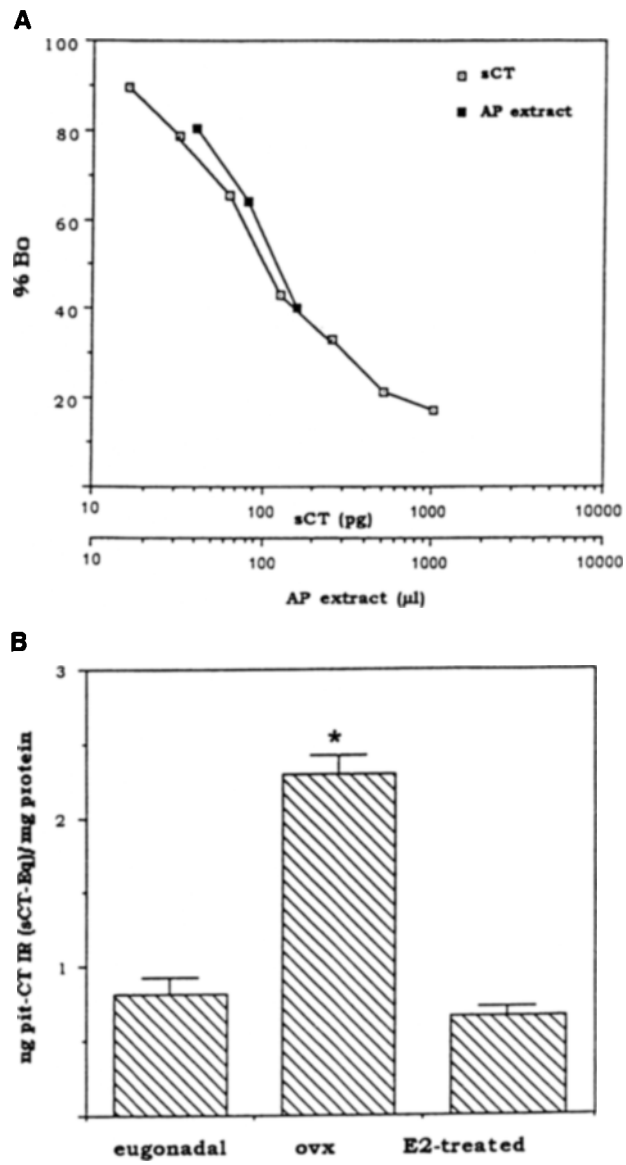

Figure 3 Effect of ovarian hormonal manipulations on pit-CT IR content in the AP gland. (**A**) Dilution curves of synthetic sCT and AP tissue extracts were examined for parallelism in a RIA. (**B**) Concentrations of pit-CT IR content in AP tissue extracts (prepared as described in the Methods section). The data are expressed as ng pit-CT IR (sCT-Eq) per mg protein. The results were statistically evaluated by *t*-tests and significance was derived from a two-tailed table. $^*P<0.001$

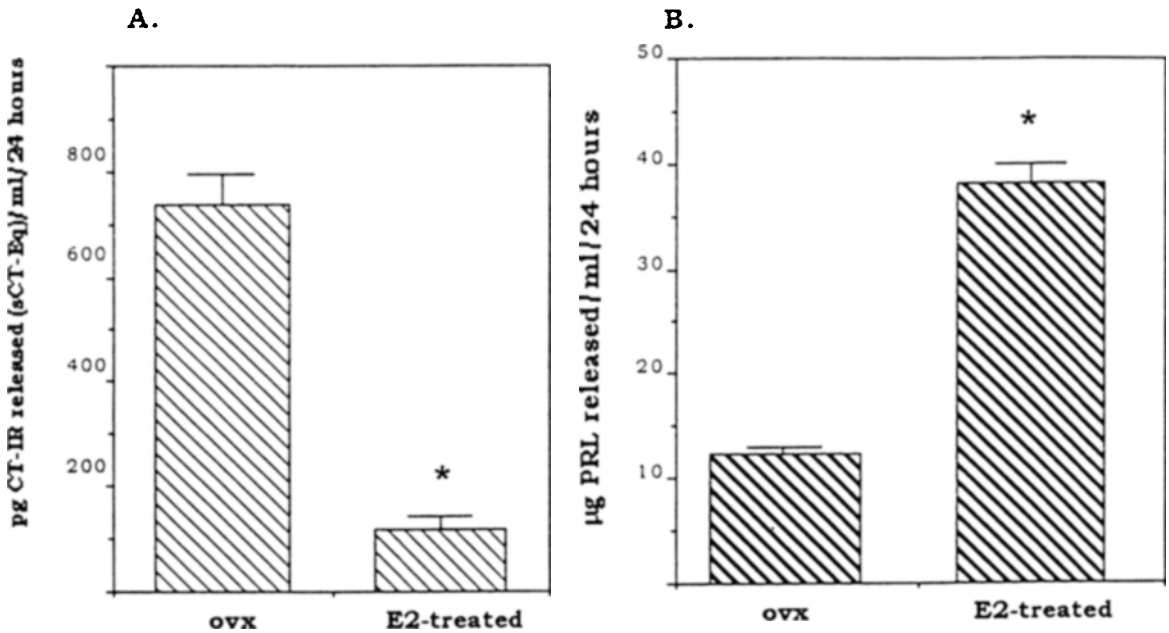

Figure 4 Release of pit-CT IR and PRL by cultured AP cells from ovx and E2-treated rats. (A) Accumulated pit-CT IR levels in conditioned media from cultures of AP cells from ovx and E2-treated rats. The results are expressed as pg pit-CT IR released/10^6 cells/24 h. Results were statistically evaluated by *t*-test and significance was derived from a two-tailed table. *$P<0.005$. (B) Accumulated PRL levels in conditioned media from the experiment described in 4A. The results are expressed as µg PRL released/ml/24 h. $P<0.01$ (*t*-test)

rols, whereas the same in AP cells from estradiol-replaced rats was only 8%. Again, AP cells from E2-treated rats exhibited over twofold higher basal PRL release as compared to those from ovx rats.

Discussion

The present results demonstrate that withdrawal of ovarian hormones induces a large increase in synthesis and release of pit-CT-IR in the AP gland, and this pattern negatively correlates with PRL secretion. Immunocytochemical studies have shown that ovariectomy induced a dramatic increase in pit-CT IR cell population of the AP gland. Moreover, these cells were distributed throughout the AP gland with no specific regional localization. In contrast, pit-CT IR cell population in eugonadal and E2-replaced rats contained significantly fewer cells. It is conceivable that withdrawal of ovarian hormones may have induced pit-CT IR expression in cell populations that previously contained undetectable pit-CT IR. Furthermore, the findings that only three days of estradiol treatment dramatically reduced pit-CT IR cell population suggest that estrogens play a major inhibitory role in the expression of pit-CT IR expression in the AP gland. These hormonal manipulations also produced similar changes in pit-CT IR content and pit-CT IR release from cultured AP cells. These results suggest that rapid effects of ovariectomy and estradiol replacement on pit-CT IR expression may occur at the level of peptide synthesis and gene expression. Since pit-CT IR expression in the present studies was immunologically determined, it could be argued that this may just reflect the uptake of exogenously produced peptide by the AP gland. However, previous and present findings, that cultured AP cells release immunoprecipitable pit-CT IR in conditioned media and its immunoneutralization with anti-sCT IgG stimulates PRL release, strengthen the case for its pituitary origin and its paracrine actions (Shah *et al.*, 1993).

Estrogens have been shown to induce major changes in AP cell function as indicated by a significant increase in PRL gene expression and synthesis as well as inhibition of LH synthesis and release under certain conditions (Amara *et al.*,

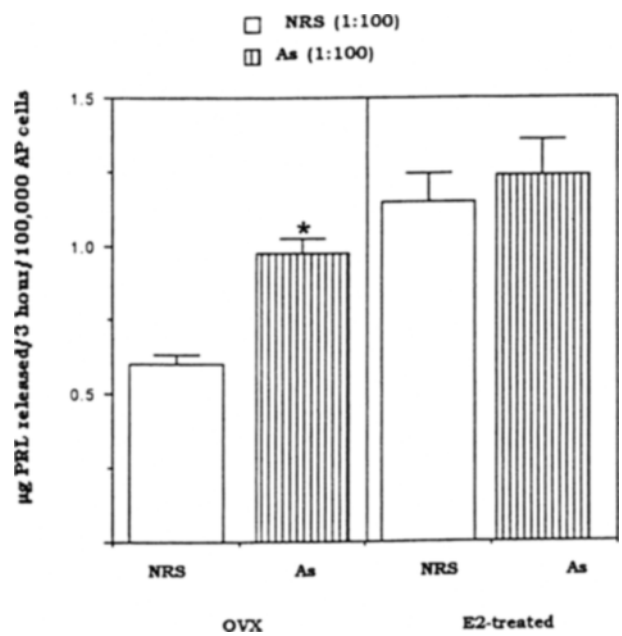

Figure 5 Effect of CT immunoneutralization on PRL release. Either anti-sCT IgG (1:100) or normal rabbit serum IgG (NRS-IgG) was added to AP cell cultures (100 000 cells per well) from ovx and E2-treated rats. Accumulated PRL levels in conditioned media were determined after three hour incubations. The results are expressed as µg PRL released/3 h/100 000 AP cells. The results were statistically evaluated by two-way ANOVA. Levels of significance: ovx *v* E2-treated: $P<0.05$ (two way ANOVA and Newman-keuls test). NRS *v* As-sCT: $P<0.05$ (two way ANOVA and Newman-keuls test). *NRS *v* As-sCT $P<0.05$ (one way ANOVA)

1987; Bhatnagar *et al.*, 1993; Castelo Branco *et al.*, 1993; Woller *et al.*, 1993). Recent evidence has shown that at least some of these actions seem to be mediated by various paracrine peptides such as VIP, galanin, neurotensin, substance P and neuropeptide Y (Frank *et al.*, 1984; Prysor-Jones *et al.*,

1989; O'Halloran *et al.*, 1990; Woller *et al.*, 1993; Wynik *et al.*, 1993). Galanin, whose expression is induced by estrogens, is produced by lactotrophs and has recently been shown to mediate estrogen-induced PRL release and lactotroph proliferation (Kaplan *et al.*, 1988; Wynik *et al.*, 1993). VIP, another lactotroph-derived autocrine factor (Hagen *et al.*, 1986; Nagy *et al.*, 1988), has also been suggested to mediate similar actions of estrogens on lactotrophs (Prysor-Jones *et al.*, 1989). The present results demonstrate that estrogens dramatically inhibit pit-CT IR expression in the AP gland. Since pit-CT is a potent inhibitor of PRL gene transcription and release, it is likely that stimulatory actions of estradiol on lactotrophs may partly be mediated through withdrawal of this inhibitory influence. The present results, that anti-sCT IgG fails to stimulate PRL release in estradiol-treated AP cells, further reinforce this possibility. Although a role for paracrine factors in regulation of PRL secretion has been suggested, these factors are also implicated in proliferation of lactotrophs (Prysor-Jones *et al.*, 1989; Wynik *et al.*, 1993). It is likely that these peptides may also serve as modulators to amplify or diminish responsiveness of AP cells to hypothalamic hormones. CT has been shown to inhibit TRH-induced PRL gene expression and release from AP cells (Shah *et al.*, 1988, 1990; Judd *et al.*, 1990). Thus, it is likely that pit-CT may serve as a paracrine inhibitor of PRL release and may also modulate the responsiveness of lactotrophs to various hypothalamic hormones. Thus, the secretion of antagonistic peptides such as galanin and VIP on one hand, and pit-CT on the other hand would provide a mechanism through which the AP gland may discriminate between various signal inputs, and regulate secretion of PRL according to physiological needs.

In summary, present results suggest that estrogens induce a rapid attenuation of pit-CT IR expression in the AP gland, and this action of estrogens is consistent with their stimulatory actions on PRL gene expression. Thus, pit-CT expression in the AP gland may vary with estrogen status of the animal and may contribute to alterations in secretory activities of lactotrophs. However, the next goal of this study remains to obtain a cDNA probe for pit-CT IR. The availability of cDNA for this peptide will help identify cellular origin of this peptide by *in situ* hybridization, and will allow us to further investigate a physiological role for this factor in the AP gland.

Materials and methods

Synthetic peptides' salmon (s) CT, human (h) CT, rat CT gene-related peptides (α and β) were obtained from Peninsula Laboratories, Belmont, CA. Culture media and other tissue culture reagents such as Trypsin-EDTA solution, Penicillin G-Streptomycin mixture, horse and fetal calf sera were obtained from GIBCO Laboratories (Grand Island, NY). FITC-conjugated goat anti-rabbit IgG was purchased Zymed Laboratories (San Fransisco, CA). All other chemicals, unless otherwise stated, were obtained from Sigma Chemical Co. (St. Louis, MO).

Animals

Adult Holtzman female rats (60–90 days old) were purchased from Harlan Inc. (Milwaukee, WI) and housed two to a cage. The animals were maintained under conditions of 12 h of light and 12 h of darkness (lights on at 0600 h) with *ad libitum* access to Purina rat chow (Ralston Purina, St. Louis, MO) and tap water. After a 4-day period of acclimatization, the rats were bilaterally ovariectomized under ketamine anesthesia. Ovariectomized rats were allowed to recover for 10 days and then implanted with a silastic tube (30 mm long, id 1.57 mm, od 3.18 mm containing 500 μg/ml 17-β estradiol propionate dissolved in sesame oil or sesame oil [vehicle]) under the skin of the back. Three days after the implantation, age-matched E2-treated and ovx rats were sacrificed to obtain the pituitary glands. The treatment has been shown to produce serum E_2 concentrations similar to those observed in the afternoon of proestrus (Wise *et al.*, 1981; Barkan *et al.*, 1983; Pilotte *et al.*, 1984). Euthanasia was performed by decapitation under ketamine anesthesia. Trunk blood samples from the rats were collected and serum was separated and stored in aliquots at -70°C. Protocols for the surgery as well as euthanasia have been approved by the Animal Care Committee at the University of Kansas Medical Center.

Immunocytochemistry (ICC)

The AP glands from experimental rats (eugonadal, ovx or E2-treated) were obtained and immediately fixed in Zamboni's fixative for 2 h, rinsed in PBS and stored overnight in 30% sucrose at 4°C (Zamboni & De Martino, 1967). The pituitaries were then frozen by submersion in isopentane-dry CO_2 bath after mounting in the embedding medium (Tissue-Tek, Miles Laboratories, Elkhart, IN). The frozen tissues were sliced to 8–10 μm thick sections and thaw-mounted on Superfrost plus glass slides (Fisher Scientific, Pittsburgh, PA). The sections were stored frozen at -70°C until ICC analysis.

Preparation of Anti-sCT IgG The present studies used IgG fraction of anti-sCT serum (GCT1-3) for ICC and immunoneutralization studies. Preparation and characterization of this antiserum has been previously described (Shah *et al.*, 1989). In brief, rabbits were immunized with synthetic sCT-keyhole limpet hemocyanin conjugate coupled at lysine residues (positions 11 and 18 from N-terminal of sCT). The anti-serum may recognize N-terminal region of the peptide that contains cystein residues at positions 1 and 7). This is because treatment of sCT with dithiothreitol (100 nM) caused a dramatic decline in its ability to bind to the GCT1-3 anti-sCT serum. Specificity of the antiserum was examined by testing its cross-reactivity with various CT-like and hypothalamic peptides. The anti-sCT serum did not cross-react with b-endorphin, met-enkephalinamide, GHRH, VIP, EGF, somatostatin, glucagon, LHRH, PHI, TRH or other CT-related peptides such as rat CT and CGRP in a sCT RIA when tested at several concentrations ranging from 1 ng to 1 μg (Shah *et al.*, 1989).

To increase specificity of the signals and to reduce non-specific serum effects, we purified anti-sCT serum (GCT1-3) further to obtain IgG fraction. This was done by first desalting the antiserum, followed by purification on Econo-Pac DEAE Blue cartridge (Bio Rad, Hercules, CA). The manufacturer's protocol was followed during the entire procedure. The eluted IgG fraction was lyophilized, resolubilized and aliquoted for subsequent use.

Immunofluorescence

Zamboni-fixed AP tissue slices or dispersed AP cells were washed several times with PBS and incubated in the blocking solution (10% normal goat serum in 0.4% triton. X-100-PBS) for 10 min at room temperature. This was followed by incubation with the primary antibody (purified rabbit anti-sCT serum, 1:100; diluted in the blocking buffer) for 48 h at 4°C. After three 5 min washes in PBS at room temperature, the cells were incubated with fluorescine (FITC)-conjugated goat anti rabbit IgG (Zymed Laboratories, San Fransisco, CA; diluted 1:50 in the blocking buffer) for 3 h at room temperature. The sections were then washed three times in PBS and mounted. The immunostaining was visualized in a Nikon Optiphot microscope equipped with filters XF 23 for fluorescein and XF32 for rhodamine (Omega Optical, Brattleboro, VT) and a 100 W high pressure mercury bulb.

The specificity of pit-CT-staining was tested in two sets of concurrent controls: (1) rabbit anti-sCT IgG was replaced with equivalent amount of nonimmune rabbit IgG, and (2)

rabbit anti-sCT IgG was preincubated with either 1 μM sCT, rCGRP-a or rCGRP-b at 37°C for 1 h prior to the use in ICC.

Cell Counts The number of pit-CT-IR containaing cells was determined at 200 × magnification by counting fluorescent cells with visible nuclei in a whole microscopic field. At least 20 fields from each specimen were counted to determine average frequency of pit-CT IR cells per field.

Tissue extraction

AP glands were separated from neural lobe and homogenized in 0.1 M acetic acid at 4°C. The homogenates were then heated at 85°C for 5 min, freeze-thawed in acetone-dry CO_2 bath and centrifuged at 36 000 r.p.m. for 30 min at 4°C. The supernatants were collected and stored frozen at −70°C until assayed. The samples were neutralized and pit-CT IR was quantitated by sCT RIA as previously described (Shah *et al.*, 1989). Since pit-CT has been shown to cross-react with anti-sCT serum, dilution curve of AP tissue extracts was tested for parallelism with standard sCT in the RIA. The detection range of the RIA for pit-CT-IR is from 14–1000 pg (sCT-Eq), and intra-assay co-efficient was 6–8%. All the samples were assayed in duplicates in a single assay to avoid interassay variation. Protein concentration of the extracts was determined by BCA method (Pierce Chemicals, Rockford, IL). The results are expressed as ng pit-CT IR/mg protein.

Pit-CT release by cultured AP cells: cell dispersal and culture

The AP glands from ovx and E2-treated rats were obtained, minced and enzymically dispersed to obtain a single cell suspension as previously described (Shah *et al.*, 1993). The dispersed AP cells were counted and checked for viability by trypan blue exclusion. Dispersed AP cells routinely exhibited 95% or greater viability. Approximately 1×10^6 cells in 1 ml culture medium were then plated per well of 24-well culture plates and allowed to attach for 48 h at 37°C in 95% air-5% CO_2 atmosphere. The cells were then washed in a serum-free basal medium and incubated in a chemically defined serum-free DMEM for 24 h. The conditioned media were collected and analysed for pit-CT IR and PRL by specific RIAs. PRL RIA used rPRL RIA kit provided by the NIDDK and the recommended directions were followed. Pit-CT IR levels were assayed by sCT RIA as described above. The results are expressed either as ng PRL or pg pit-CT IR (sCT-Eq) released 1×10^6 AP cells/24 h.

Biological activity of pit-CT: effect of anti-sCT IgG on PRL release

The AP glands from ovx and E2-treated rats was enzymically dispersed separately, and the cells were plated at a density of 100 000 cells per well in a 96-well culture plate. The cells were cultured in 250 μl medium (chemically defined serum-free DMEM containing 1 nM 17-β estradiol or vehicle for AP cells from E2-treated and ovx rats respectively) for 2 days under standard conditions (Shah *et al.*, 1993). On third day of the culture, the cells were washed in basal RPMI medium (containing 0.3% BSA, 10 mM HEPES 2.2 mM bacitracin) and incubated either with non-immune rabbit IgG or rabbit anti-sCT IgG (at a dilution of 1:50) for 3 h. Each treatment was carried out in quadruplicate. At the end of the incubation, conditioned media were collected and stored frozen at −70°C until analysed for PRL content by RIA. The PRL RIA used protein A (IgG sorb, Malden, MA) for separation of bound hormone from the free, and therefore the presence of anti-sCT IgG in the samples did not interfere with the assay. Each experiment was repeated at least four different times and the results are expressed as mean ng PRL released ± SEM per 100 000 cells per 3 h.

Statistics Where appropriate, the results were statistically evaluated by either *t*-tests or ANOVA as described in the results.

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