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# Calcitonin-Like Peptide Containing Gonadotrophs Are Juxtaposed to Cup-Shaped Lactotrophs

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**Calcitonin (CT) is known to inhibit basal and TRH-stimulated prolactin release in cultured anterior pituitary cells in vitro and pituitary CT-like peptide (pit-CT) is synthesized and released by isolated anterior pituitary cells. However, the specific cell type containing pit-CT has not been identified. To determine this, double label immunohistochemistry was performed on pituitary sections from male rats using antisera for specific marker peptides of gonadotrophs, thyrotrophs, lactotrophs, somatotrophs, corticotrophs, and folliculo-stellate cells. CT was only colocalized with gonadotroph-specific markers and the distribution of pit-CT immunoreactive (IR) cells followed the patterns of gonadotroph distribution in male and female rats. Double and triple label immunohistochemistry using antiserum for CT, FSH, and PRL showed an apposition of calcitonin-like peptide containing gonadotrophs to cup-shaped lactotrophs. To examine whether pit-CT IR was altered, similarly to gonadotrophs, with known changes in PRL serum levels, studies were extended to ovariectomized, pregnant, and lactating rats. The area covered by pit-CT immunoreactivity and the tissue content of pit-CT significantly differed between physiological states and the pit-CT level was inversely related to the known PRL status. Pit-CT containing gonadotrophs were in all cases apposed to cup-shaped lactotrophs. These results provide histological support for previous studies proposing that pit-CT serves as a paracrine inhibitor of PRL release.**

**Key Words:** Pituitary; colocalization; gonadotrophs; calcitonin; paracrine; rat.

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## Introduction

Calcitonin (CT) is a 32-amino acid peptide secreted by the thyroid gland. In addition, CT or related peptide(s) are expressed in a number of tissues, including the anterior pituitary gland (Potts and Aurbach, 1976; Sexton and Hilton, 1992). Exogenously added CT selectively and potently inhibits basal and TRH-stimulated PRL gene expression and release from rat anterior pituitary cells (Shah et al., 1988; Judd et al. 1990). The action of CT is specific; it does not affect basal GH, TSH, FSH, or LH release and neither GnRH-induced LH release nor TRH-induced TSH release are altered (Shah et al., 1990). Pituitary calcitonin-like peptide (pit-CT) is synthesized and released by anterior pituitary cells (Shah et al., 1993), although the specific cell type has not been identified. The sequence of pit-CT has not been determined, but seems to share antigenic sites with salmon (s) and human (h) CTs. IgGs directed against these peptides immunoprecipitate similar molecular species from anterior pituitary cell lysates, label a specific, yet unknown, cell population of the gland, and significantly increase PRL release when added to cell cultures. These results show that pit-CT may serve as a paracrine inhibitor of PRL release (Shah et al., 1993).

Increasing evidence demonstrates that cell-to-cell interactions, including direct intercellular contacts through gap junctions (Denef and Andries, 1983) or secretion of paracrine peptides (Denef et al., 1986), play important roles regulating PRL secretion (Horvath et al., 1977). Lactotrophs comprise two morphologically different subpopulations: polygonal or ovoid-shaped, which are not associated with other cell types and cup-shaped, which are frequently apposed to gonadotrophs (Nakane, 1970,1975; Horvath et al., 1977; Sato, 1980; Allaerts et al., 1991). An inverse relationship between lactotroph and gonadotroph secretion has been observed (Ben-David et al., 1971; Denef et al., 1986), implying paracrine interactions.

The objective of the present study was to characterize the anterior pituitary cell type(s) containing pit-CT immunoreactivity (pit-CT IR) and to test if the histological relationship between pit-CT IR cells and lactotrophs is

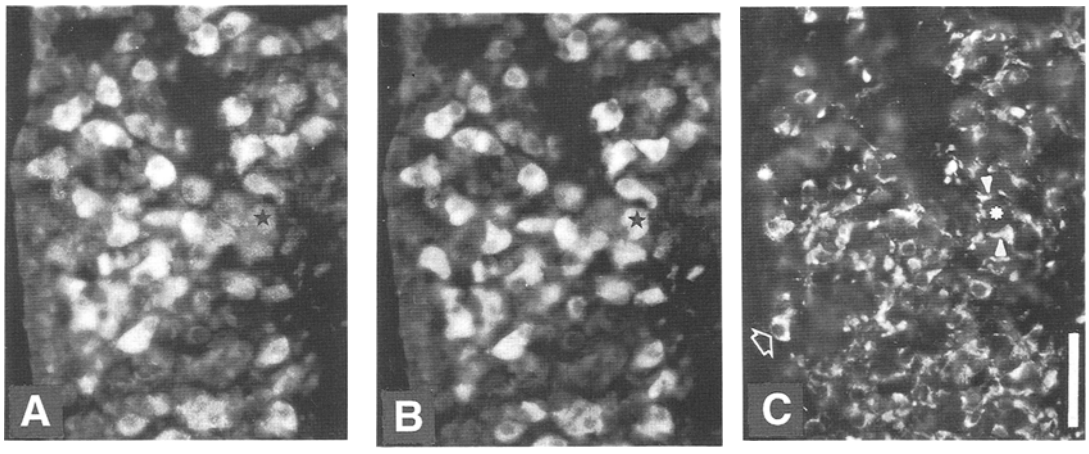


**Fig. 1.** Section from the periphery of a male pituitary anterior lobe showing triple label immunohistochemistry clearly localizing pituitary calcitonin-like peptide immunoreactivity (pit-CT IR) in gonadotrophs. **(A)** pit-CT rhodamine labeled secondary antiserum; **(B)** FSH, fluorescein label; and **(C)** PRL, AMCA label. Black stars in A and B indicate a gonadotroph, the position of which is marked by an asterisk in C. The gonadotroph (asterisk in C) is surrounded by cup-shaped lactotrophs (white arrow heads in C). Open arrow in C indicates a polygonal lactotroph. ×240; bar 50 μm.

consistent with previously proposed paracrine interactions. Immunohistochemical colocalization experiments demonstrated pit-CT IR in gonadotrophs juxtaposed to lactotrophs. Pit-CT IR cells, similarly to gonadotrophs, were distributed in distinctly different patterns in male and female rats. Additional studies, using immunohistochemistry and radioimmunoassay (RIA), were designed to study whether pit-CT IR cell populations are modulated in ovariectomized, pregnant, and lactating rats.

## Results

### *Cellular Localization of Pit-CT IR Determined by Double-Label Immunohistochemistry*

Double label fluorescent immunohistochemistry showed that pit-CT IR was colocalized with FSH IR (Fig. 1A,B) in 98% of 1600 FSH IR cells in male rats, in 97% of 1100 FSH IR cells in ovariectomized rats, in 99% of 1200 FSH IR cells in pregnant rats, and in 97% of 1100 FSH IR cells in lactating rats. Similar results were obtained with LH IR. Sections double stained for pit-CT IR and FSH IR and adjacent sections stained for LH demonstrated that the three antigens were colocalized in 95% of gonadotrophs, implying that most gonadotrophs secrete both FSH and LH. The pit-CT IR was highly granular with the intensity of immunoreactivity varying among individual gonadotrophs within one gland (Fig. 2D).

TSH and α-MSH were found in polyhedral cells, S-100 was present in stellate-shaped cells, and GH in small oval cells. PRL immunoreactivity was found in polygonal and cup-shaped cells, two morphologically distinct populations of lactotrophs. TSH was located in central regions of the lobe, whereas PRL, GH, α-MSH, and S-100 containing cells were found throughout the lobe. None of these antigens were colocalized with pit-CT IR. Thus, pit-CT IR was exclusively localized in gonadotrophs.

### *Pit-CT IR Cells and Their Apposition to Lactotrophs*

Almost all cells containing pit-CT IR (95% in the male, 96% in ovariectomized rats, 96% in pregnant rats, and 95% in lactating rats) were apposed to cup-shaped lactotrophs (Fig. 3A–D). Pit-CT IR cells were surrounded by one or several lactotroph nuclei situated in a small cytoplasmic area that extended into thin processes partly embracing the gonadotroph. The pit-CT cells apposing cup-shaped lactotrophs also contained FSH (Fig. 1A–C).

PRL IR appeared more intense in polygonal lactotrophs than in cup-shaped, possibly because of the visual impact of the larger cytoplasmic area of the polygonal cell present (Fig. 3B,D). In male rats, the majority (80%) of lactotrophs were cup-shaped. In ovariectomized rats, polygonal lactotrophs were more numerous, but still constituted a minority (40%). In contrast, polygonal lactotrophs dominated in the pregnant and lactating rat (90%). Thus, the cup-shaped lactotroph population was largest in the male gland and progressively declined in the glands of ovariectomized, pregnant, and lactating rats. In contrast, the polygonal lactotroph population was smallest in the male gland and successively increased in the glands of ovariectomized, pregnant, and lactating rats. The increasing numbers of polygonal lactotrophs in the paradigms were accompanied by an overall increase in PRL IR intensity.

### *Pit-CT IR Cells in Various Physiological Conditions*

In male rats, most pit-CT IR cells were localized around the periphery of the lobe (Fig. 2A), with a few cells dispersed in the central region (Fig. 2B), consistent with the distribution of gonadotrophs. In ovariectomized rats, the cells were more evenly distributed throughout the lobe with the peripheral to central gradient not as pronounced (Fig. 2C,D). In pregnant rats, many pit-CT IR cells were located along the periphery of the lobe (Fig. 2E), though many were

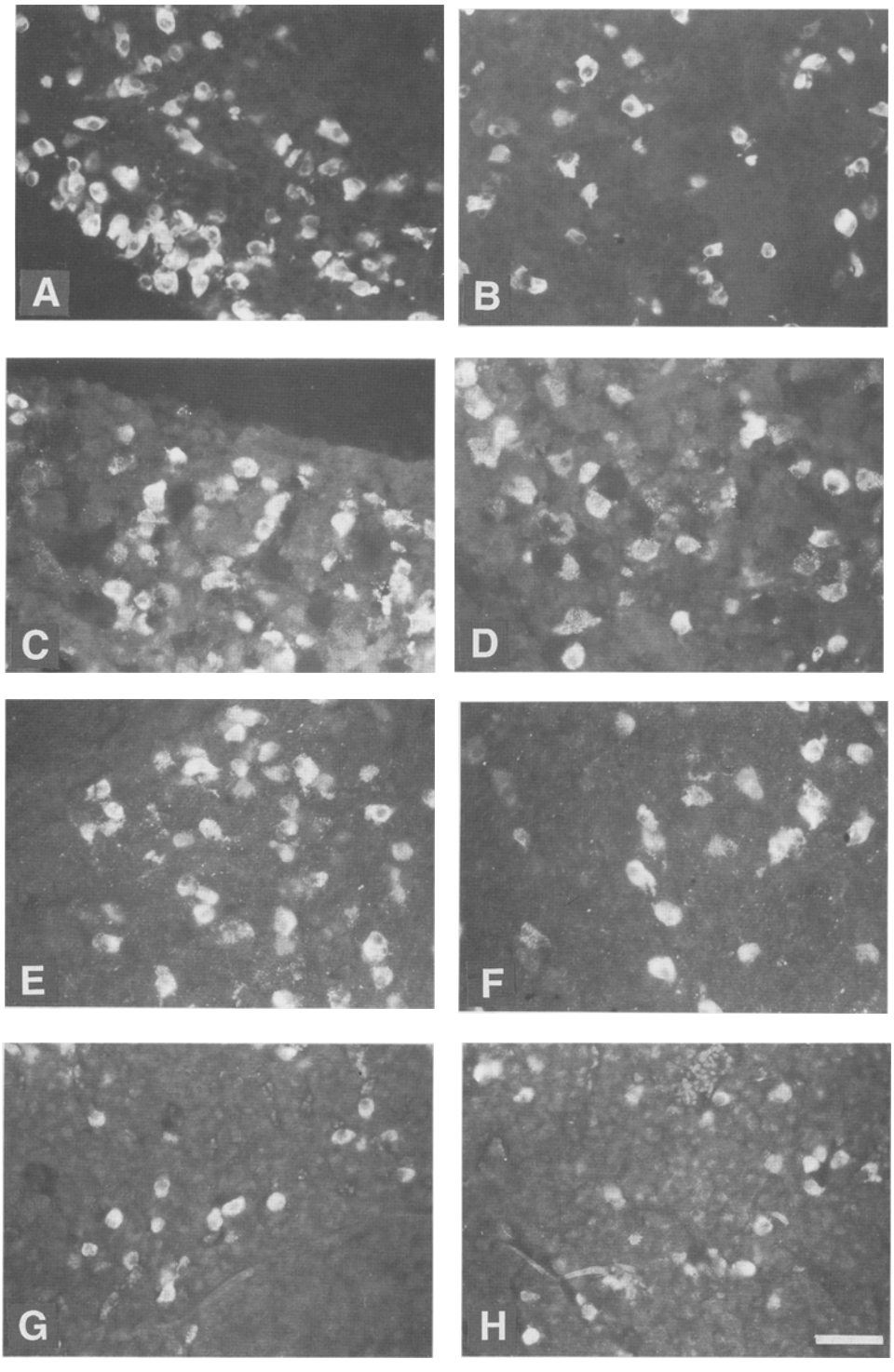


**Fig. 2.** Comparison of pit-CT IR in peripheral **(A,C,E,G)** and central **(B,D,F,H)** parts of anterior pituitary glands from (A,B) male; (C,D) ovariectomized; (E,F) pregnant (gestation d 20); and (G,H) lactating (d 10) rats. In the male rat, most pit-CT IR cells were localized in the periphery. In the ovariectomized rat, cells were more evenly distributed; in pregnant rats, there was a gradient between periphery and central parts, whereas in lactating rats pit-CT IR cells were evenly distributed. ×190; bar 50 μm.

also distributed in central parts (Fig. 2F). Thus, the gradient was more pronounced than in ovariectomized, but not as apparent as in male rats. There was no difference in distribution between gestation d 16 and 20. In lactating rats, pit-CT IR cells were evenly distributed throughout the lobe similarly to the ovariectomized rats (Fig. 2G,H). The intensity of pit-CT IR changed with the duration of lactation. At d 2 of lactation, pit-CT IR was barely detectable above background; by d 10, the intensity approached the levels observed in male, ovariectomized, and pregnant rats.

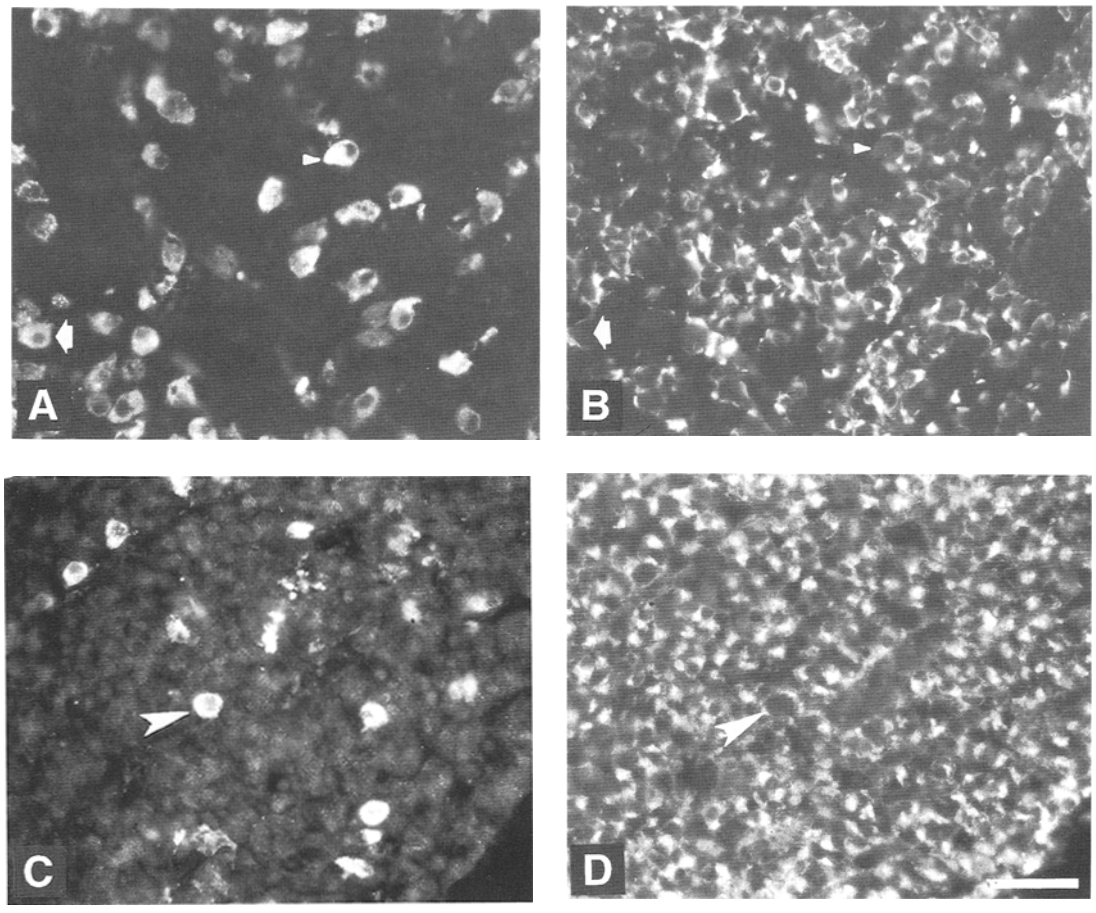


**Fig. 3.** Double label immunohistochemistry shows pit-CT immunoreactive gonadotrophs **(A,C)** in apposition to cup-shaped PRL immunoreactive lactotrophs **(B,D)**. In the male (A and B), the majority of lactotrophs were cup-shaped with pit-CT IR cells numerous and apposed to cup-shaped lactotrophs (at arrows and arrow-heads in A and B). In d 10 lactating rat (C and D), polygonal lactotrophs dominated and pit-CT IR cells were few, however, still apposed to cup-shaped lactotrophs. ×240; bar 50 μm.

### *Gonadotroph Cross-Section Area in Various Physiological Conditions*

Gonadotrophs immunoreactive for LH covered 4.6 ± 0.1% of the section area of the anterior lobe of male rats, while in ovariectomized rats, the area was significantly larger (6.1 ± 0.2%) than in any other treatment ($p < 0.001$). The increased area could have resulted from cellular hypertrophy, because these gonadotrophs appeared larger (Fig. 2B), and/or from an increased number of cells expressing the gonadotroph-specific marker. Likely, both hypertrophy and hyperplasia occurred, as has been demonstrated for LH-IR gonadotrophs following ovariectomy (Gross, 1978). In pregnant rats, gonadotrophs covered 3.5 ± 0.1% of the section, which was significantly less ($p < 0.001$) than in male and ovariectomized rats, while in d 2 lactating rats, gonadotrophs covered 2.6 ± 0.1%, a significantly ($p < 0.001$) smaller area than in the other paradigms. By d 14 of lactation, the area covered increased significantly (3.1 ± 0.2; $p < 0.001$), but was still significantly smaller than in pregnant rats in agreement with tissue levels of LH by radioimmunoassay (Merchant, 1974). We observed similar changes using fluorescent immunohistochemistry for pit-CT, however, for technical reasons, LH IR, detected by PAP, had to be used as a marker for image analysis.

### *Anterior Lobe Content of Pit-CT in Various Physiological Conditions*

RIA measured the anterior lobe content of pit-CT to be 2.31 ± 0.12 (mean ± SEM) ng sCT-Eq/mg protein in ovariectomized rat (10 d postsurgery); 0.54 ± 0.03 in pregnant rat (gestation d 20), 0.07 ± 0.01 on lactation d 2, and 0.32 ± 0.02 on lactation d 14. These data corroborated the immunohistochemistry data. Peptide contents of pregnant and lactating rats were significantly different from that of ovariectomized at $p < 0.03$ and $p < 0.01$, respectively. Peptide contents were significantly different between lactation d 2 and 14 ($p < 0.03$). Values from lactating d 12 were significantly different from those of pregnant rats ($p < 0.05$;), whereas those from d 14 were not.

## Discussion

The present study demonstrated, using immunohistochemical colocalization methods, that gonadotrophs were immunoreactive for the pit-CT-like peptide. In the male,

pit-CT IR cells were preferentially distributed along the periphery of the gland whereas they were more evenly distributed in the female, consistent with the known distribution of gonadotrophs (Denef and Andries, 1983). Further, pit-CT IR cells were surrounded by cup-shaped lactotrophs.

Gonadotrophs are selectively apposed to cup-shaped lactotrophs (Nakane, 1970, 1975; Horvath et al., 1977; Sato, 1980; Allaerts, 1991). Cell-to-cell interactions may be critical for normal development and growth of these cell types. Differentiation of lactotrophs is induced by the LH-α subunit (Begeot et al., 1994), although this is debated (Horacek et al., 1989). Both cup-shaped and polygonal lactotrophs are mature, but the cup-shaped are morphologically further differentiated and their apposition to gonadotrophs may indicate a functional relationship with these cells (Sato, 1980). Selective ablation of gonadotrophs leads to nonfunctional or dysfunctional lactotrophs (Kendall et al., 1991) and hereditary PRL and GH deficiencies result in diminished gonadotroph function and lowered fertility (Tang et al., 1993). Elevated PRL secretion attenuates LH secretion (Winters and Loriaux, 1978; Cheung, 1983; Smith and Bartke, 1987) as well as responsiveness of gonadotrophs to LHRH (Cheung, 1983). Gonadotrophs may influence PRL release through secretion of paracrine factors because there is no evidence for a direct effect of FSH and LH on PRL secretion. Angiotensin II is released by gonadotrophs in response to LHRH and may mediate LHRH-induced PRL release (Jones et al., 1988). Calcitonin inhibits basal and TRH-stimulated PRL release and has been proposed to function as a paracrine factor (Shah et al., 1989, 1990); the localization of pit-CT in gonadotrophs provides the morphological basis for this functional relationship.

Although there is biochemical evidence for interactions between lactotrophs and gonadotrophs, there is little information on pituitary gonadotropin and PRL levels in relation to histological apposition between gonadotrophs and lactotrophs. In the present study, a small pit-CT IR cell population was associated with glands from conditions with high PRL secretion, such as early lactation or pregnancy. In contrast, glands from rats which secrete lower amounts of PRL, such as the male (MacLeod et al., 1965) and the ovariectomized female (Amenomori et al., 1967), had a larger pit-CT IR cell population. Thus, the pit-CT IR cell population was inversely related to the level of PRL secretion. Moreover, in pregnant and lactating rats, most PRL IR cells were oval or polygonal, whereas cup-shaped lactotrophs were barely detectable. PRL IR intensity was highest in these rats, which may be owing to hypertrophy reflective of enhanced PRL production in the polygonal lactotrophs (Sato, 1980). This is further supported by the finding that PRL is at its highest level in the pituitary in early lactation as found by RIA (Merchant, 1974). In the male gland, pit-CT IR cells were preferentially located in the periphery, where also the majority of cup-shaped lactotrophs are distributed (Salo, 1980). Only a small percentage of the lactotrophs were polygonal, implying that the association of cup-shaped lactotrophs with gonadotrophs and the influence of juxtacrine pit-CT may be mechanisms for lowering PRL secretion. In contrast, conversion of lactotrophs to polygonal shape accompanied by dissociation from gonadotrophs and lack of exposure to pit-CT would increase PRL secretion. If this assumption is true, then hormones that increase prolactin secretion would also increase the proportion of polygonal lactotrophs to cup-shaped cells. Estradiol, which is a potent inducer of PRL secretion (Giguere et al., 1982, Franks, 1983) and gene expression (Vician et al., 1979; Shupnik et al., 1979; Shull and Gorski, 1990), reduces the population of cup-shaped lactotrophs (Allaerts et al., 1991). $E_2$ also attenuates pit-CT expression in the anterior pituitary gland (Li and Shah, 1995). This supports the possibility that prolactin secretagogues may convert lactotrophs from low-secreting cup-shaped to high-secreting polygonal-shaped.

Our results demonstrated that pit-CT is located in gonadotrophs in close apposition to lactotrophs, thus providing the morphological basis for a paracrine interaction between pit-CT cells and lactotrophs. This could be a mechanism through which the anterior pituitary gland regulates PRL secretion according to physiological needs.

## Methods

### *Animals*

Male (150–175 g) and timed-pregnant Sprague-Dawley rats were obtained from Sasco, Inc. (Omaha, NE) and were housed 2 to a cage on a 12/12 h light/dark cycle with free access to food and water. Females were sacrificed either 14 or 20 d into gestation or after 2, 10, or 14 d of lactation. Adult female rats were bilaterally ovariectomized under ketamine anesthesia and allowed to recover for 10 d. Rats were euthanizied by decapitation under ketamine anesthesia (KU animal protocol 91-06-07-00 for surgery and euthanasia).

### *Preparation of Tissues for Histology*

Pituitaries were removed from the basal cranium, immersed in periodate-lysine-paraformaldehyde fixative (0.01*M* $NaIO_4$, 0.075*M* lysine, 0.0375*M* $NaPO_4$ buffer, pH 7.4; and 2% paraformaldehyde) overnight, cryoprotected in 25% sucrose overnight, frozen on dry ice, and embedded in OCT compound (Miles, Elkhart, IN). Pituitaries were cryostat-sectioned at 14 μm and sections thaw-mounted onto subbed slides.

### *Preparation of Pit-CT Antiserum*

Preparation and characterization of this antiserum (GCT1-3) has been previously described (Shah et al., 1989). In brief, rabbits were immunized with synthetic salmon calcitonin-keyhole limpet hemocyanin coupled to lysine residues in positions 11 and 18 from the N-terminal. The antiserum did not cross-react with LH, FSH, various hypo-

thalamic peptides, or calcitonin-related peptides such as rat calcitonin and CGRP in a salmon calcitonin RIA when tested at concentrations ranging from 1 ng to 1 μg (Shah et al., 1989).

The detection range for the RIA, was 7–1000 pg. To increase specificity of the signals, the antiserum was further purified to obtain the IgG fraction by desalting followed by purification on Econo-Pac DEAE Blue cartridge (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. The eluted IgG fraction was lyophilized, resolubilized, and aliquoted for subsequent use.

### *Fluorescence Immunohistochemistry*

Slides were equilibrated in phosphate-buffered saline with 0.2% Triton X-100 (PBS-TX) for5 min and placed in primary antiserum overnight at 4°C. The following antisera were used: rabbit anti-sCT (1:50; [5]), mouse antirat PRL (1:3500, Dr. J. Schammel, University of South Alabama), mouse anti-S-100 (1:300, Chemicon, Temecula, CA), monkey anti-GH (1:5000, NIH, Bethesda, MD), guinea pig anti-LH (1:100, NIH, Bethesda, MD), guinea pig anti-FSH (1:100, NIH, Bethesda, MD), rabbit anti-TSH (1:100, NIH, Bethesda, MD), and sheep anti-α-MSH (1:1000, Dr. J. Tatro, Tufts University, Boston, MA). Slides were washed three times for 5 min each in PBS-TX, and placed in the appropriate secondary antiserum for 1.5 h at room temperature in a light-sealed moist chamber. The following secondary antisera were used: fluorescein conjugated goat antimonkey IgG (1:300, Jackson Immunoresearch, West Grove, PA), fluorescein conjugated donkey antirabbit IgG (1:300, Jackson), rhodamine conjugated donkey antimouse IgG (1:300, Jackson), rhodamine conjugated goat antiguinea pig IgG (1:300, Organon Teknika/Cappel, Durham, NC), AMCA conjugated donkey antisheep IgG (1:150, Jackson), and AMCA conjugated goat antimouse IgG (1:150, Jackson). Slides were then washed three times for 5 min each in PBS-TX. For double-label immunohistochemistry, slides were placed in the second primary antiserum and the procedure was repeated as described. For triple labeling, the slides were placed in the third primary antiserum and the procedure was repeated again. Slides were coverslipped with aqueous mounting medium (1:1 mixture of glycerol/PBS). Preabsorption of the pit-CT antiserum with pit-CT peptide obliterated immunohistochemical staining. Other controls included omitting the second or third primary antiserum. There was no cross-reactivity with the donkey and goat secondary antisera; they could be used interchangeably. However, double-label combinations including sheep anti-αMSH were exceptions, since only donkey secondary antiserum could be used. Three-to-five animals per group were used for these experiments. Preparations to be compared were processed simultaneously. Two researchers independently evaluated the slides, scoring all slides at the same time to avoid comparing preparations that had been stored or exposed to UV-light for different periods of time.

### *Peroxidase Anti-Peroxidase (PAP) Immunohistochemistry*

All steps with the exception of the 3,3-diaminobenzidine (DAB) preincubation and reaction were performed at 4°C. Sections were pretreated with 3% $H_2O_2$ in absolute methanol (1:4) for 5 min to inactivate endogenous peroxidases, 10% normial goat serum for 15 min to block nonspecific binding of secondary antibodies, and then placed in primary antiserum overnight (rabbit anti-sCT-like peptide, or guinea pig anti-LH). Slides were washed three times in 0.1*M* $NaPO_4$ buffer for 10 min each, and incubated for 30 min in a moist chamber with unconjugated goat anti-rabbit IgG or goat antiguinea pig IgG (Cappel) (1:100). The buffer rinses were repeated and then slides were incubated for 30 min in a moist chamber with rabbit PAP (Cappel) or mouse or guinea pig PAP (Jackson Immunochemicals) (1:100). Slides were washed three times for 10 min each in 0.1*M* $NaPO_4$ buffer, and incubated for 10 min in 0.05% DAB (Sigma) in 0.1*M* Tris-saline at room temperature. After the 10-min incubation, $H_2O_2$ was added to the DAB solution (to 0.001% final concentration), mixed, and slides incubated for 10 min. The reaction was stopped with PBS and then distilled water. Controls included omission of primary antiserum and replacement of primary antiserum with normal serum. This method provided the permanent preparations used for area measurements.

### *Cell Counts and Area Measurements*

The incidence of colocalization of pit-CT IR and FSH or LH immunoreactivities was determined using double fluorescence immunohistochemistry. Three sections from comparable rostro-caudal levels of male, ovariectomized, pregnant, and lactating rats were evaluated (three animals per group); 300–400 cells were counted per tissue section. The percentage of pit-CT IR cells apposed to cup-shaped PRL immunoreactive lactotrophs was evaluated in double-label fluorescence immunohistochemical preparations.

Since pit-CT PAP immunohistochemistry was less than optimal, the percent anterior pituitary area covered by gonadotrophs was quantitated using LH PAP immunohistochemistry. The image analysis system consisted of a Dage/MTI model 72 CCD camera mounted on the trinocular port of a Zeiss Axioplan microscope. The camera was connected to a Matrox MVP-AT array processor installed in a 486-based AT bus PC with 80486 math coprocessor, 44 MB Bernoulli box, and a 160-MB hard disk drive running commercial software (MicroMeasure FL-4000, Georgia Instruments, Roswell, GA). The threshold was adjusted to include only LH IR gonadotrophs for area measurements. Two researchers independently made the measurements on slides with covered labels All values were expressed as the mean ± SEM and were evaluated by one-way analysis of variance (ANOVA) followed by the Bonferroni posthoc test (GraphPad InStat, San Diego, CA).

### *Tissue Extraction and RIA*

Anterior pituitary 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 an acetone-dry $CO_2$ bath, and centrifuged at 100,000*g* 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 crossreact with anti-sCT serum, a 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 to 1000 pg (sCT-Eq), and intra-assay coefficient was 6–8%. All samples were assayed in duplicates in a single assay to avoid interassay variation. Protein concentration of the extracts was determined by the BCA method (Pierce Chemicals, Rockford, IL). The results are expressed as ng sCT Eq/mg protein and analyzed by Student's *t*-test. Data are presented as mean ± SEM for $n = 3$.

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