Effects of Hyperprolactinemia on Calcitonin Secretion in Male Rats

Chien-Chen Lu, Shiow-Chwen Tsai, William J.S. Huang, Ching-Lin Tsai, and Paulus S. Wang

The role of prolactin (PRL) in calcitonin (CT) release by the thyroid C cell in male rats was studied. Anterior pituitary (AP)-grafted male rats were characterized by hyperprolactinemia. Brain cortex (CX)-grafted male rats were used as control animals. AP- and CX-grafted rats were infused intravenously with $CaCl_2$ and bled from the jugular catheter at 0, 30, 60, and 120 minutes following the $CaCl_2$ challenge. Rat thyroid gland was incubated with or without 3-isobutyl-1-methylxanthine (IBMX) at 37°C for 30 minutes. Thyroid C cells were incubated in culture medium at 37°C for 60 minutes. Cyclic adenosine 3′,5′-monophosphate (cAMP) in rat thyroid tissues following incubation with IBMX was extracted by 65% ethanol. AP-grafted rats had higher plasma levels of PRL and CT compared with CX-grafted rats. Both the release of CT and accumulation of cAMP in thyroid glands were higher in AP-grafted versus CX-grafted rats. Direct administration of ovine PRL (oPRL) on the thyroid glands did not increase CT secretion in vitro. Thyroid C cells of AP-grafted rats secreted more CT compared with CX-grafted rat cells. These results suggest that hyperprolactinemia increases the release of CT by thyroid C cells in rats through a cAMP-dependent pathway caused by an indirect effect of PRL.

THE ANTERIOR PITUITARY (AP)-grafted male rat has provided an experimental model of hyperprolactinemia to study endocrine function.[1] Prolactin (PRL) excess is associated with disorders of calcium metabolism,[2-4] especially bone loss,[4] in humans. Plasma levels of growth hormone (GH), thyrotropin, thyroxine, triiodothyronine, and testosterone are normal in the AP-implanted male rat.[1,5] PRL has been shown to stimulate calcium absorption from the gut[6] and to enhance 1,25-dihydroxyvitamin D[1,25-$(OH)_2$D] production in rats.[7]

The changes in calcitonin (CT) secretion in hyperprolactinemic rats have been seldom investigated. Clinical reports show that basal plasma CT levels are slightly reduced in hyperprolactinemic women, and calcium-stimulated plasma CT is normal in the hyperprolactinemic state and similar to CT levels during bromocryptine treatment.[8] Meanwhile, serum levels of CT decrease in Buffalo rats bearing the MMQ tumor (from the 7315a rat pituitary tumor), which secretes large amounts of PRL and GH, and urinary calcium excretion increases as the tumors grow, although serum calcium levels and parathyroid hormone, and urinary cyclic adenosine 3′,5′-monophosphate (cAMP) excretion were normal in tumor-bearing rats.[9]

Plasma levels of PRL in humans[7] and rats[10,11] increase by age. Age-related changes in CT levels are reported in both humans[12,13] and rats.[14-17] There is a progressive decrease of plasma CT with age in humans, and it is possible that aging itself may generally decrease the secretory capacity of C cells.[12] Hypercalcitoninemia occurs in aged rats,[14-17] and the increased secretion of CT is probably due to β-adrenergic affectors,[15] an aging-related decline in estrogen secretion,[16] or an alteration in regulation by calcium.[17] Serum calcium itself does not change, but hormones that regulate calcium metabolism change markedly with age.[17-19] However, the mechanisms of hypercalcitoninemia regulated by aging still are not clear.

The present study was designed to investigate the effects of hyperprolactinemia on CT secretion both in vivo and in vitro in rats. The adenylate cyclase system has been implicated in CT secretion in previous studies,[20] and the proportion of C cells to follicular cells is 10.4% in 4-month-old rats.[21] The hyperprolactinemic effects on cAMP accumulation in rat thyroid glands were examined to test whether cAMP production is involved in the regulation of CT secretion in hyperprolactinemic rats. We also studied the effect of hyperprolactinemia on CT secretion by thyroid C cells. We found that the greater secretion of CT in AP-grafted rats is at least due to a cAMP-associated hypersecretion of CT by the thyroid gland.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 350 to 400 g were housed in a temperature-controlled (22° ± 1°C) room with 14 hours of artificial illumination daily (6 AM to 8 PM) and provided with food and water ad libitum.

Implantations were performed under ether anesthesia. The rats were implanted with AP or brain cortex ([CX] control) under the capsule of the kidney.[22] Six weeks after implantation, plasma PRL increased in AP-grafted rats, but not in CX-grafted rats. These hyperprolactinemic rats were used for the experiments.

In Vivo Experiments

Rats were catheterized via the right jugular vein and left femoral vein[23] prior to a challenge with $CaCl_2$ (10 mg/mL) at a rate of 1 mL/30 min.[16] The infusion was performed with the femoral catheter connected to a peristaltic pump. Blood samples (0.6 mL each) were collected from right jugular vein at 0, 30, 60, and 120 minutes following the challenge.[16]

The plasma was separated by centrifugation at 10,000 × *g* for 1 minute and stored at −20°C for radioimmunoassay (RIA) of CT. Plasma calcium was determined by an automatic calcium analyzer (Calcette; Precision Systems, Natick, MA).

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In Vitro Experiments

After decapitation, rat blood samples were collected, and CT and PRL plasma levels were measured by RIA. Rat thyroparathyroid glands were excised, bisected, and preincubated with Locke's solution containing 10 mmol/L glucose, 0.003% bacitracin, and 0.05% HEPES at 37°C for 90 minutes.[24] Thyroparathyroid glands were then incubated with vehicle (Locke's medium) or ovine PRL ([oPRL] 40 ng/mL) for 30 minutes. At the end of incubation, the tissue was weighed and the medium was collected and measured for CT by RIA.

For studying the correlation between CT and accumulation of cAMP in vitro, the thyroparathyroid glands of rats were excised, bisected, and incubated with medium containing 1 mmol/L 3-isobutyl-1-methylxanthine (IBMX), a phosphodiesterase inhibitor. One hemithyroid was assigned to a tube. At the end of incubation, tissues were mixed with 1 mL 65% ice-cold ethanol, homogenized by a polytron (PT-3000; Kinematica, Luzern, Switzerland), and centrifuged at 10,000 × *g* for 3 minutes.[25,26] The supernatants were lyophilized by a vacuum concentrator (SpeedVac; Savant Instruments, Holbrook, NY) and reconstituted with assay buffer (0.05 mol/L acetate buffer with 0.01% azide, pH 6.2) before measuring the cAMP concentration by RIA.[25] The protein concentration in tissue extracts was determined by Lowry's method.[27]

Thyroid C Cell Culture

Thyroid glands obtained from AP- and CX-grafted rats were minced and digested with collagenase (1 mg/mL) in Dulbecco's phosphate-buffered saline (PBS) at 37°C for 60 or 150 minutes as previously described.[28] The isolated thyroid C cells were then preincubated in a 12 × 75 mm plastic tube for 60 minutes in 0.5 mL (per thyroid) medium 199 with Earle salts supplemented with nonessential amino acids, 2 mmol/L glutamine, 10% dextran charcoal-coated fetal bovine serum, 125 U/mL penicillin, and 100 μg/mL streptomycin. Two to four tubes of cells were plated from each thyroid gland. The rest of the isolated thyroid C cells were transferred to a 15-mL conical centrifuge tube and centrifuged for 5 minutes at 200 × *g*. The pellet was resuspended in 0.5 mL 0.7% bovine serum albumin (BSA)-PBS. The supernatant was added on the top of a 15-mL conical centrifuge tube with discontinuous percoll gradient buffers (density range, 1.00 to 1.10 g/mL) and centrifuged for 20 minutes at 1,100 × *g*. The crude cell suspension was divided into four layers. Each layer was transferred to a 15-mL conical centrifuge tube and washed twice with 10× PBS buffers. The cells were then preincubated in culture medium at 37°C for 60 minutes. After preincubation, all cells were incubated in culture medium at 37°C for 60 minutes. At the end of incubation, the cells were centrifuged for 5 minutes at 200 × *g*. The supernatants were collected and measured for CT by RIA. The pellet was washed with 2 mL PBS buffers. The protein concentration in the pellet was determined by Lowry's method.[27]

RIA of CT

The CT concentration in plasma and medium samples was measured by a human CT RIA kit[16,29] purchased from Nichols Institute Diagnostics (San Juan Capistrano, CA). The inhibition curves of rat thyroid medium and plasma or human plasma were plotted and shown to be parallel to the standard curve of human CT. The antisera for CT showed virtually no cross-reactivity against bovine parathyroid hormone (PTH)-(1-84), human PTH-(1-34), insulin, PRL, human growth hormone, thyrotropin, and corticotropin. Salmon CT up to 40 ng/mL did not cross-react with the antisera. The sensitivity was 4 pg/mL. CT recovery from human serum pools was 86% to 94%. The intraassay and interassay coefficients of variation were 6.7% (n = 10) and 8.3% (n = 10), respectively.

RIA of PRL

PRL concentrations in the plasma samples were determined by RIA as described previously.[23,30] The rat PRL kit was provided by the National Institute of Diabetes and Digestive and Kidney Diseases. Rat PRL-I-9 was used for radioiodination and rat PRL-RP-3 was the standard. The sensitivity of the rat PRL RIA was 3 pg per assay tube. The intraassay and interassay coefficients of variation were 3.8% and 3.2% for the PRL RIA, respectively.

RIA of cAMP

The thyroid cAMP concentration was determined by RIA as described previously.[25,30] With the anti-cAMP serum no. CV-27 pool, cAMP sensitivity was 2 fmol per assay tube. The intraassay and interassay coefficients of variation were 6.9% (n = 5) and 11.9% (n = 5), respectively.

Statistical Analysis

All values are presented as the mean ± SEM. For the in vitro studies, the treatment means were tested for homogeneity using ANOVA of variance, and the difference between specific means was tested for significance using Duncan's multiple-range test.[31] For the in vivo studies, Student's *t* test was used. A difference between two means was considered statistically significant at a *P* level less than .05.

RESULTS

Basal Plasma Levels of PRL, Calcium, and CT

The plasma PRL concentration was higher in AP-grafted versus CX-grafted rats ($P < .05$). There were no significant differences in basal plasma calcium between AP- and CX-

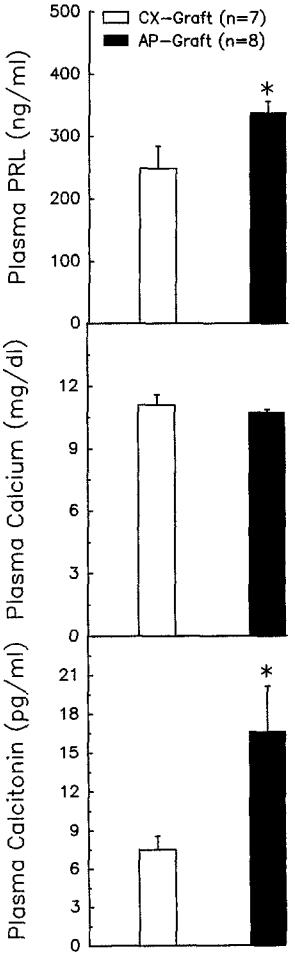

Fig 1. Basal plasma PRL, calcium, and CT in male rats grafted with CX or AP. *$P < .05$ *v* CX-grafted rats.

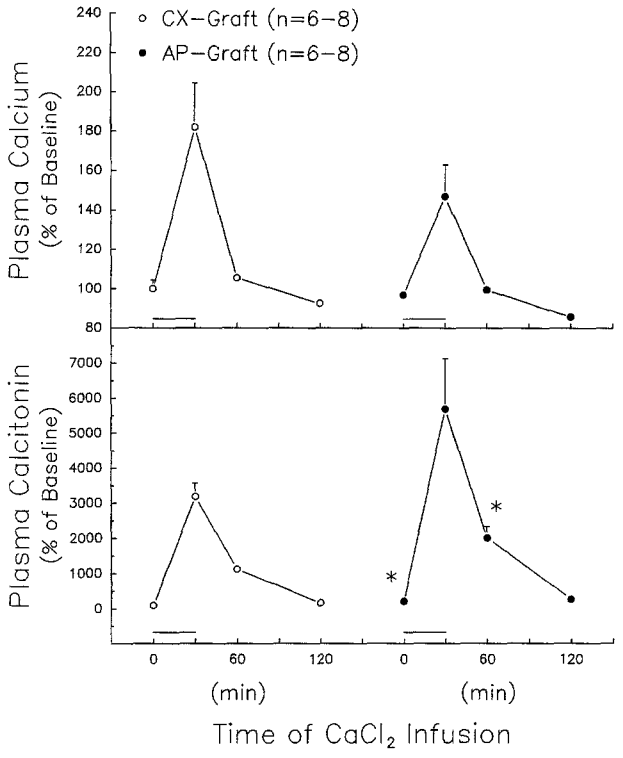

Fig 2. Effect of intravenous infusion of $CaCl_2$ on plasma calcium and CT in male rats grafted with CX or AP. Rats were infused intravenously with $CaCl_2$ (30 mg/kg) from 0 to 30 minutes (horizontal line) via a peristaltic pump (1 mL/30 min). Values are expressed as the percentage increase over basal levels of plasma calcium or CT in CX-grafted rats. *$P < .05$ *v* CX-grafted rats.

grafted rats. The basal plasma level of CT was higher in AP-grafted rats than in CX-grafted rats (122%, $P < .05$; Fig 1).

Response of CT to $CaCl_2$ Challenge

The percentile change in plasma calcium and CT levels in response to intravenous infusion of $CaCl_2$ is illustrated in Fig 2. There were no significant differences in post-$CaCl_2$ plasma calcium levels between AP- and CX-grafted rats. After 30 minutes of infusion of $CaCl_2$, plasma calcium increased in all groups. Infusion of $CaCl_2$ for 30 minutes increased the plasma concentration of CT in all rats. Thirty minutes after termination of $CaCl_2$ infusion, rat plasma CT was higher in AP-grafted versus CX-grafted rats.

Effects of Hyperprolactinemia on CT Release and cAMP Accumulation In Vitro

In vitro release of CT from the thyroid gland was greater in AP-grafted versus CX-grafted rats ($P < .01$; Fig 3). The production of thyroid cAMP in response to IBMX in AP-grafted rats was higher than in CX-grafted rats ($P < .01$; Fig 3).

Effects of oPRL on CT Release and cAMP Accumulation In Vitro

Administration of oPRL did not alter the in vitro release of thyroid CT or accumulation of thyroid cAMP in male rats (Fig 4).

Effects of Hyperprolactinemia on CT Release by Thyroid C Cells

In vitro release of CT by thyroid C cells following enzymatic dispersion for either 60 or 150 minutes was greater ($P < .01$) in AP-grafted versus CX-grafted rats (Fig 5).

Effects of Hyperprolactinemia on CT Release by Thyroid C Cells From Different Layers of Percoll Gradient

In vitro release of CT by thyroid C cells from layer II and layer III (density 1.02 and 1.03, respectively) was higher in AP-grafted compared with CX-grafted rats. CT release from layer IV (mostly red blood cells, density 1.07) was almost undetectable in CX- and AP-grafted groups (Fig 6).

DISCUSSION

The present results demonstrate that compared with CX-grafted rats, (1) AP-grafted rats possess higher levels of plasma

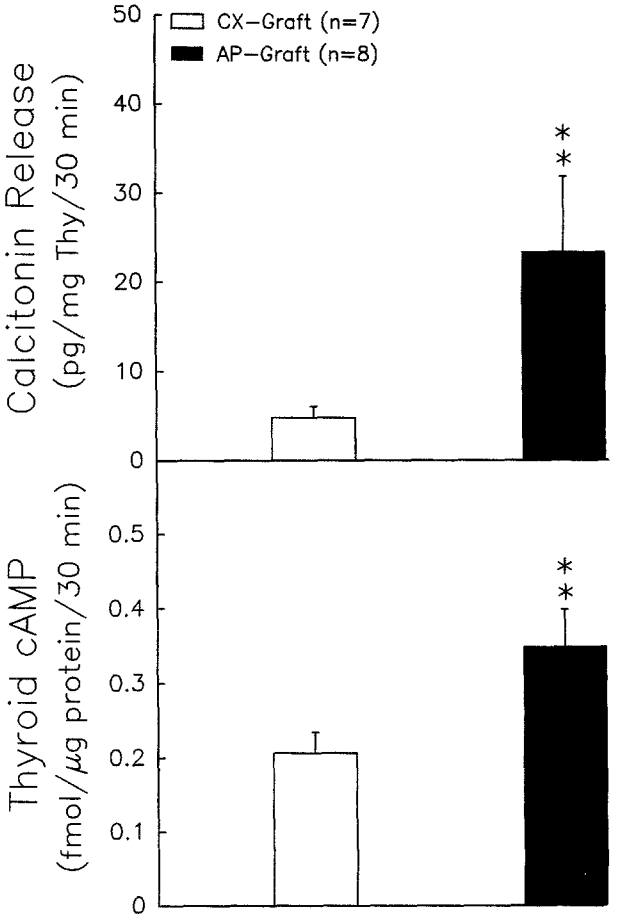

Fig 3. In vitro release of CT and accumulation of cAMP in thyroid gland of AP- and CX-grafted rats. Rat thyroid tissue was preincubated and then incubated with Locke's medium at 37°C for 30 minutes. One hemithyroid was assigned per tube. At the end of incubation, the tissue was weighed, and the medium was collected and measured for CT by RIA. For studying cAMP accumulation, thyroparathyroid glands were incubated with the medium containing 1 mmol/L IBMX, a phosphodiesterase inhibitor, at 37°C for 30 minutes. Each hemithyroid was then extracted by 1 mL 65% ethanol before measurement of cAMP by RIA. **$P < .01$ *v* CX-grafted rats.

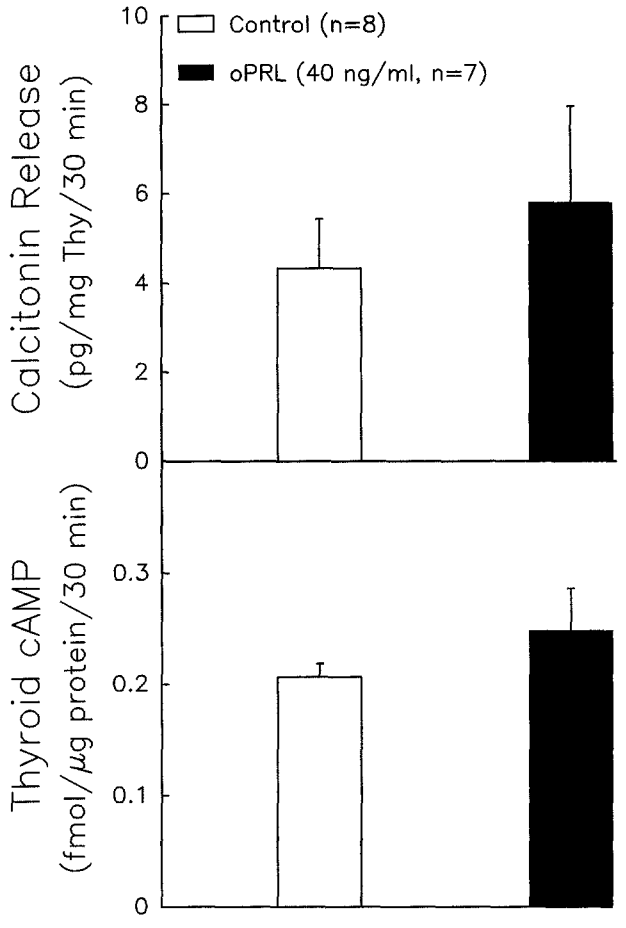

Fig 4. Effect of oPRL in vitro on CT release and cAMP accumulation in rat thyroid glands. Refer to Fig 3 for details.

PRL and CT, (2) thyroid CT release and cAMP accumulation were greater in AP-grafted rats, and (3) thyroid C cells of AP-grafted rats secret more CT.

The hyperprolactinemic syndrome can be induced by AP engraftment,[22] MMQ tumor,[9] dopamine antagonist agents,[32] PRL injection,[33] estrogen treatment,[34] or aging.[10,11] The effects of hyperprolactinemia on plasma CT have only been reported by Adler et al.[9] A model of chronic hyperprolactinemia, the MMQ tumor–bearing Buffalo rat, has lower serum levels of CT,[9] a phenomenon that may be due to large amounts of PRL (about the 10-fold level in the male rats) and GH, and urinary calcium excretion increases as the tumor grows daily.[9] In addition, MMQ tumor grows every day, and the plasma level of PRL is also increased. Thus, it may produce hypogonadism and hypercalciuria. These effects may cause low plasma CT levels in MMQ tumor–bearing rats. The hyperprolactinemia observed with MMQ tumor does not increase plasma CT levels, so it does not explain the hypercalcitoninemia in aged rats. Thus, another model of hyperprolactinemia was used to study the effects of aging on CT secretion.

Hyperprolactinemia is associated with decreased bone mineral density in humans[4,8] and induces a lower basal serum level of testosterone but a higher response to human chorionic gonadotropin (hCG).[35] Basal plasma CT levels are slightly reduced in hyperprolactinemic women, but the CT response to

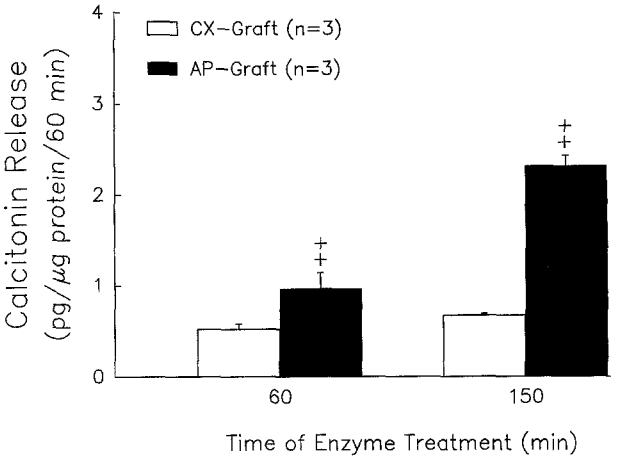

Fig 5. Effect of hyperprolactinemia on CT release in vitro from the thyroid C cell. Thyroid glands were minced and digested with collagenase (1 mg/mL) in Dulbecco's PBS at 37°C for 60 or 150 minutes. Isolated thyroid C cells were preincubated and then incubated for 60 minutes in 0.5 mL medium 199 with Earle salts supplemented with nonessential amino acids, 2 mmol/L glutamine, 10% dextran charcoal-coated fetal bovine serum, 125 U/mL penicillin, and 100 μg/mL streptomycin. At the end of incubation, the cells were centrifuged for 5 minutes at 200 × *g*. The supernatants were collected and measured for CT by RIA. The pellet was washed with 2 mL PBS buffers. The protein concentration in the pellet was determined by Lowry's method. ††*P* < .01 *v* CX-grafted rats.

calcium is normal.[8] Foresta et al[36] found that androgen deficiency per se plays an important role in the pathogenesis of osteoporosis in hypogonadal subjects, and suggested that androgens may influence bone metabolism by regulating CT secre-

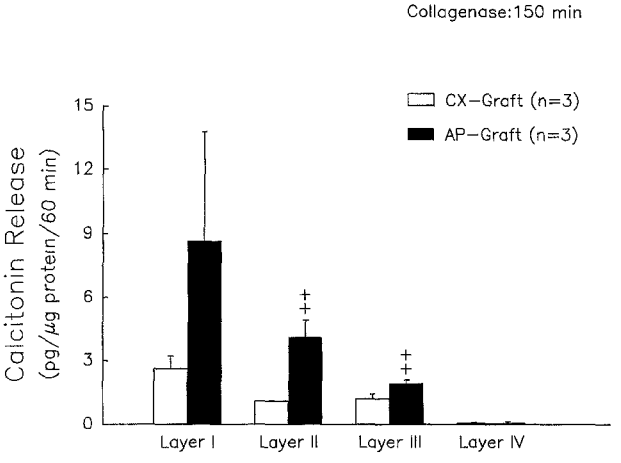

Fig 6. Effect of hyperprolactinemia on CT release in vitro from thyroid C cells in different layers of percoll gradient. Isolated thyroid C cells were transferred to a 15-mL conical centrifuge tube and centrifuged for 5 minutes at 200 × *g*. The pellet was resuspended in 0.5 mL 0.7% BSA-PBS. The supernatant was added on the top of a 15-mL conical centrifuge tube with discontinuous percoll gradient buffers (density range, 1.02-1.10 g/mL) and centrifuged for 20 minutes at 1,100 × *g*. The crude cell suspension was separated into 4 layers. Each layer was transferred to a 15-mL conical centrifuge tube and washed twice with 10× PBS buffers. Cells were preincubated and then incubated in culture medium at 37°C for 60 minutes. At the end of incubation, the cells were centrifuged for 5 minutes at 200 × *g*. Supernatants were collected and measured for CT by RIA. The protein concentration in the pellet was determined by Lowry's method. ††*P* < .01 *v* CX-grafted rats.

tion. Therefore, hyperprolactinemia may decrease plasma CT through a mechanism associated with androgen deficiency in humans.

Previous results demonstrated that the hyperprolactinemic rat induced by AP implantation has normal plasma levels of GH, thyrotropin, thyroxine, and triiodothyronine.[1-5] In our study, AP-grafted rats have high plasma PRL and CT but normal plasma calcium before and after calcium challenge. CT release from the thyroid gland was increased in AP-grafted rats in vitro. cAMP accumulation in the thyroid gland was also increased in AP-grafted rats in vitro. Since the adenylate cyclase system has been implicated in CT secretion by previous studies[20] and the proportion of C cells to follicular cells is 10.4% in 4-month-old rats,[21] these results suggest that the increased CT secretion by hyperprolactinemia was due to increased thyroid cAMP accumulation. In addition, this is the first report of the use of primary culture of thyroid C cells from hyperprolactinemic rats to study the effect of hyperprolactinemia on CT secretion. Secretion of CT by thyroid C cells was increased in AP-grafted rats in vitro. These results suggest that C cells of the thyroid gland in AP-grafted rats were activated by chronic hyperprolactinemia. This effect may increase CT release from thyroid C cells.

The hyperprolactinemic effects are also found in aged rats. Aged rats have higher plasma PRL and CT than young rats, and serum calcium itself does not change with age.[18,37] These physiological phenomena were similar to our hyperprolactinemic model. Thus, we suggest that PRL may be a factor to increase CT release from thyroid C cells. However, incubation of PRL directly with the thyroid gland did not increase CT secretion and cAMP accumulation in vitro. Therefore, the mechanism of increased CT release from C cells of AP-grafted rats may be due to hyperprolactinemic effects but not direct effects of PRL on the thyroid gland. The hypercalcitoninemia in hyperprolactinemic rats is probably due to the production of CT-releasing factor(s) caused by PRL via a cAMP-dependent pathway.

In summary, we have demonstrated that hyperprolactinemia increased the release of CT from rat thyroid C cells via a cAMP-dependent pathway caused by an indirect effect of PRL.

ACKNOWLEDGMENT

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