Effects of Calcium-Modulating Hormones on Thiazide Receptor Density$^{1,2}$

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ABSTRACT

Thiazide diuretic drugs act in the distal convoluted tubule (DCT) to inhibit a Na$^+$-Cl$^-$ cotransporter and enhance reabsorption of luminal calcium. The density of receptors for thiazides in the rat DCT is known to be increased by adrenocortical steroids, furosemide, and bendroflumethiazide, but decreased by ischemia. Because the DCT is a physiologic site of action by calcitonin and parathyroid hormone, this study examined the effects of these calcitropic hormones in thyroparathyroidectomized Sprague-Dawley rats on (1) the density of the rat thiazide receptor (TZR), as quantitated by binding of [3H]metolazone to renal membranes, and (2) urinary electrolyte excretion rate. Salmon calcitonin (sCT) (20 to 100 ng/h) (1) increased the density of the renal TZR twofold, an effect that is maximal by 6 h after sCT administration, and (2) decreased urinary calcium excretion rate. Adequate dietary calcium must be provided for the effects of sCT to be observed. Regression analysis demonstrated that renal TZR density correlated negatively with total urinary calcium excretion rate but not with plasma calcium ion concentration. In addition, neither rat calcitonin (rCT), at doses that cause hypocalcemia, nor parathyroid hormone, at doses that cause hypercalcemia, produce direct effects on TZR density in the DCT of the thyroparathyroidectomized rat. Our findings indicate that upregulation of TZR by sCT, which occurs independently of plasma calcium ion concentration, is likely via a calcitonin-like receptor other than that for rat calcitonin itself.

Key Words: Calcitonin, thiazides, calcium excretion

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2 A portion of the data from this study was presented in abstract form at the American Society of Nephrology Annual Meeting in Orlando, FL, October 26-29, 1994.
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The distal nephron, comprised of the distal convoluted tubule (DCT), the connecting tubule and the collecting duct, plays a key role in the net total body balance of calcium. Specifically, the DCT reabsorbs 8 to 10% of the filtered load of calcium (1). This reabsorption occurs against a chemical and electrical gradient (1). Physiological regulation of tubular calcium reabsorption occurs in the DCT and/or connecting tubule segments and can be dissociated from Na reabsorption with amiloride and thiazide diuretic drugs or after hormonal modification by parathyroid hormone (PTH) (1--3). Thiazide diuretics are an important class of drugs that inhibit an apical Na$^+$-Cl$^-$ symporter and at the same time enhance calcium reabsorption (4). Rat thiazide drug receptors can be quantitated by the ability of renal membranes to bind [3H]metolazone, a potent diuretic with a thiazide-like mechanism of action (5). These receptors have been shown to be under regulation. Furosemide (6), bendroflumethiazide (7) and adrenocortical steroid (8,9) administration increase TZR density whereas only 10 min of ischemia results in decreased [3H]metolazone binding (10).

Parathyroid hormone (PTH) and calcitonin (CT) act to regulate the amount of calcium in the urine at the level of the DCT in most species. The major renal effects of PTH in the rat are phosphaturia and increased renal calcium reabsorption (11). The effects of CT on urinary calcium excretion rate have been ambiguous with reports of increased (12) and decreased (13,14) calcium excretion rate. Recent studies suggest that CT increases calcium reabsorption (15,16). In the rat, these effects of CT have been elicited by using salmon CT (sCT), human CT, or porcine CT. However, there are no reports in the literature showing an effect of administered rat CT (rCT) on renal excretion of calcium in the rat.

In this paper, we examine the effect of rat and salmon CT and rat PTH on the (1) density of the TZR, by quantifying the maximal binding of [3H]metolazone to renal membranes, and (2) urinary electrolyte excretion in thyroparathyroidectomized (TPTX) Sprague-Dawley rats.

METHODS

Animals

Intact, sham, and TPTX Sprague-Dawley male rats were purchased from Charles River Laboratories, Wilmington, MA. One study, mentioned in the Results section, used male rats of the Wistar-Kyoto strain (WKY). All study protocols were approved by the Animal Subjects Committee of the University of California, San Diego. All animals were maintained in the American Association for Accreditation of Laboratory Animal Care-approved animal care facility and were provided

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free access to tap water and to rodent chow (Diet 8604 with 1.45% calcium; Harlan Teklad, Madison, WI) unless indicated otherwise.

Hormones

The hormones were purchased from Sigma Chemical Co. St Louis, MO. CT was diluted in water and L-α-aminoacaproic acid, pH 5.5, and administered via intraperitoneal (IP) injection or Alzet® osmotic minipump in the doses indicated in the individual experiments in the Results section. Rat synthetic PTH (1–34) was diluted in water, adjusted to pH 5.6, and given IP or by minipump. Thyroxine was diluted in 10^{-5} M sodium hydroxide and either given IP (10 μg/day) every 3 days or placed in the drinking water (10 ng/mL or approximately 0.3 μg/day). The minipumps were placed subcutaneously in an interscapular area via a dorsal incision while rats were under halothane anesthesia. Control animals received diluent via the minipumps.

Binding of [3H]metolazone

[3H]metolazone was custom synthesized by Amer sham (Arlington Heights, IL). The binding assay for [3H]metolazone was conducted as previously described (6). In brief, whole kidneys were homogenized in 10 mL ice-cold 50 mM Tris-PO4 buffer, pH 7.4. Membranes were prepared by centrifuging them for 5 min at 600 x g and the resulting supernatant twice at 45,000 x g and the resulting supernatant at 4°C for 20 min. The final pellet was evenly suspended in 10 mL buffer and diluted to achieve a final concentration of 0.8 to 1.0 mg protein/mL in the binding assay. Binding of [3H]metolazone to each membrane preparation was quantitated at six concentrations of [3H]metolazone, ranging from 0.313 to 10 nM. The specific binding of [3H]metolazone, as defined by displacement with 10^{-4} M hydroflumethiazide, was analyzed by the method of Scatchard to calculate the density and the dissociation constant (Kd) of the binding using the Elsivier Binding Data Analysis (EBDA) program of McPherson (17). Protein was determined by the Bradford Coomassie blue method (18) with bovine gamma globulin as the standard.

Urine and Blood Assays

Urine was collected on the day of euthanasia either by gentle massage of the urinary bladder while the animal was still conscious or needle aspiration from the bladder of the anesthetized rat. Blood was collected from the tail artery or retro-orbital artery while rats were under ketamine anesthesia or via heart puncture while rats were under pentobarbital anesthesia at the time of euthanasia. All blood was collected in heparinized needles and syringes. Sodium, potassium, and ionized calcium levels were measured by ion-sensitive electrodes (NOVA) within minutes after the blood was drawn. Chloride, total calcium, and creatinine levels were measured in plasma and/or urine by colorimetry using commercially available kits (Sigma Chemical Co. St. Louis, MO.)

Statistical Analysis

Data are expressed as mean values ± the standard error of the mean. Statistical significance was assessed using the Statview™ statistical program (Abacus Concepts, Inc, Berkeley, CA). When three or more groups were compared, analysis of variance was followed by Fisher's post hoc Protected Least Significance Difference (PLSD) test for multiple comparisons. Regression analyses were conducted with the Statview™ program.

RESULTS

Effects of Calcitonin on Intact Animals

To study the effect of CT on the DCT, we initially measured TZR density in membrane preparations of kidney homogenates from intact WKY rats treated with the hormone. sCT, known for being the most potent of the CT in terms of inducing hypocalcemia, was used and compared with rCT. As shown in Table 1, 5 h after administration of sCT, the density of binding sites for [3H]metolazone had increased by more than 65% in intact WKY males. At the same dose (250 ng ip) and over the identical time span, rCT had no effect. There was no significant change in plasma or urine sodium, potassium, chloride, or calcium levels (data not shown).

Plasma calcium concentration is known to be tightly controlled by a dynamic interaction of both CT and PTH. In intact animals, acute administration of CT causes a decrease in plasma calcium concentration, which triggers PTH release from the parathyroid gland. To eliminate release of this counterregulatory hormone, TPTX animals were utilized in subsequent experiments as indicated below.

Effects of Thyroxine

TPTX animals require thyroid replacement to maintain a euthyroid state (19). To assure that the dose of thyroxine given had no effect on TZR density, a dose-response study was performed. TPTX animals were divided into three groups matched by weight, and implanted with minipumps delivering 0.3, 1.0, or 10 μg/day of thyroxine for 7 days. Sham-operated animals received minipumps with diluent. The results showed that TZR density, Kd, body and kidney weight were unchanged by thyroid replacement (data not shown). Subsequently, 0.3 μg of thyroxine was given in the drinking water.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>sCT</th>
<th>rCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Thiazide Receptor Density (pmol/mg)</td>
<td>.82 ± 0.04</td>
<td>1.37 ± 0.07</td>
<td>.82 ± 0.06</td>
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<tr>
<td>Plasma Ionized Calcium (mM)</td>
<td>1.21 ± 0.02</td>
<td>1.17 ± 0.04</td>
<td>1.20 ± 0.02</td>
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</table>

a Control rats were given an intraperitoneal bolus of diluent, and test rats were given a single 250-ng ip bolus of salmon (sCT) or rat (rCT) calcitonin. Blood was collected by heart puncture at the time of euthanasia (5 h after administration of sCT).

b Values are mean ± SE.

c Significantly different from control at P < .0001.
was without effect.

Ca-Modulating Hormones and Thiazide Receptors

... clum) for 6 days (1.45% calcium-Dawley rats were fed a high-calcium diet prior experiences of some other investigators. We note of calcium or the renal TZR, despite producing hy-

calcium excretion rate was not. Interestingly, bbe 3) confirmed the results found in intact animals: that all of the studies reporting on the effect of CT in vivo in rats, the failure of rCT to alter plasma calcium concentration and urinary excretion rate of calcium as well as the ability of rCT to lower plasma calcium concentration were expected; however, the failure of rCT to alter urinary calcium excretion rate was not. Interestingly, the failure of rCT to alter either the renal excretion rate of calcium or the renal TZR, despite producing hypercalcemia, may be consistent with the unpublished prior experiences of some other investigators. We note that all of the studies reporting on the effect of CT in vitro in rats, and on adenylate cyclase stimulation by CT in specific nephron segments from rat kidney in vitro in rats, have not utilized rCT, but have used calcitonin from salmon, human, or porcine origin (13,14,15,21-25). We do not know if some of these investors also tested for, and failed to find, an effect of rCT on renal calcium handling in the rat kidney.

Recently published information about calcitonin receptors may be informative in this regard. It is now known that there is more than one isoform of the rat calcitonin receptor. Significantly, the ligand specificities of the two forms differ. The rat C1b calcitonin receptor isoform binds sCT, but lacks the ability to bind the native rCT (26). In addition, the CT receptors are members of a family of related peptide receptors (including those for calcitonin gene-related peptide, adrenomedullin, amylin, and secretin), which have considerable crossreactivity (27). Indeed, our findings in the present experiments fit best with the interpretation that renal calcium excretion rate and TZR are regulated by a member of the calcitonin-related receptor family, which is activated by sCT but not by rCT itself. This raises the possibility that a hormone other than "classical rat calcitonin" is responsible for renal calcium conservation during hypercalcemic chal-

lenge. This speculation is currently being examined in greater detail in our laboratory (28).

Does sCT act in the DCT directly via a CT receptor in the DCT to increase renal TZR density or does sCT act in the DCT indirectly affecting other cells or organs, which in turn produce the increases in the TZR in the kidney? Two important observations are relevant to these questions. First, there appears to be no correlation between plasma ionized calcium and TZR density. Hypocalcemia alone does not increase the TZR receptor. In TPTX rats with plasma ionized calcium concentra-
tions below that of sham-operated control rats (Table 3 and 4), the TZR density did not increase significantly. With acute administration (1 day) of sCT, the plasma calcium level decreased and TZR density increased (Table 2; HCD). With chronic ad-

ministration of sCT (7 days), the renal TZR increased whereas the plasma ionized calcium level normalized (Table 3). Thus, TZR density is independent of plasma calcium. Second, there is a statistically significant

![Figure 2. Effect of salmon calcitonin (sCT) and parathyroid hormone (PTH) on renal thiazide receptor (A) and plasma ionized calcium (B). Male thyroparathyrodecomnized Sprague-Dawley rats were fed a high-calcium diet (1.45% calcium) for 6 days and given (via minipump) no hormone (N = 5), 100 ng/h sCT (N = 4), 250 ng/h PTH (N = 5), or 100 ng per h/250 ng per h sCT/PTH (N = 5) for 1 day. Renal thiazide receptor density is in pmol/mg (A). Plasma ionized calcium is in mM (8).](image)

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>TZR Density</th>
<th>Ionized Calcium</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham Control</td>
<td>5</td>
<td>0.68 ± 0.04</td>
<td>1.15 ± 0.03</td>
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<tr>
<td>TPTX Control</td>
<td>5</td>
<td>0.83 ± 0.03</td>
<td>0.81 ± 0.04</td>
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<tr>
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<td>6</td>
<td>1.15 ± 0.10</td>
<td>0.58 ± 0.01</td>
</tr>
<tr>
<td>TPTX + sCT 24 h</td>
<td>6</td>
<td>1.10 ± 0.11</td>
<td>0.68 ± 0.04</td>
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<tr>
<td>TPTX + sCT 72 h</td>
<td>6</td>
<td>1.19 ± 0.08</td>
<td>0.74 ± 0.04</td>
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Values are mean ± SE. N, number of male Sprague-Dawley rats. TPTX, thyroparathyroidectomized animals; sCT, salmon calcitonin; TZR, thiazide receptor in pmol/mg protein. Ionized calcium is in mM.

sCT was administered via osmotic minipump at 25 ng/h. Animals in the 2-h, 6-h, and 24-h groups received a single 50-ng injection at the start of the experiment.

Values are significantly different from TPTX control at P < 0.05.

Values are significantly different from TPTX control at P < 0.005.

Values are significantly different from TPTX control at P < 0.0001.
negative correlation in sCT-treated animals between the renal density of thiazide receptor and urinary calcium excretion rate (Figure 1 and Table 2). The fact that sCT causes an increase in calcium reabsorption in the rat nephron supports previously published work (13,14). Although the correlation is strong, one cannot eliminate the possibility that some or all of the decreased calcium excretion rate produced by sCT might be the result of decreased GFR or increased calcium reabsorption proximal to the DCT.

Temporarily, sCT upregulated the renal TZR within hours, as shown in Table 4. TZR density is increased 19% by 2 h (statistically not significant with the number of animals used) and is maximal by 6 h. The rapid accumulation of TZR in rat kidney after sCT may result from activation of pre-existing receptors or transporters, an alteration in the transcription of the TZR gene, increased efficiency of TZR mRNA translation, or prolongation of the half-life of the TZR protein. Further experiments are needed to distinguish between these various mechanisms.

In conclusion, the density of rat renal TZR, as quantitated by the maximal specific binding of $[^{3}H]$metolazone, increased twofold after administration of sCT to intact and TPTX animals. Regression analyses demonstrate that TZR density versus urinary calcium excretion rate correlates negatively after administration of sCT for 24 h. Ingestion of adequate calcium in the diet is necessary for these changes to be observed. rCT and PTH have no direct effect on TZR density in the DCT of the rat. We suggest that the rat TZR density is regulated by some member of the calcitonin gene family other than rCT itself.

ACKNOWLEDGMENTS

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