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Down-regulation of rat kidney calcitonin receptors by salmon calcitonin infusion evidenced by autoradiography

(renal binding sites/calcitonin infusion)

Z. BOUIZAR*†, W. H. ROSTÈNE‡, AND G. MILHAUD*

*U.113 Institut National de la Santé et de la Recherche Médicale, Centre Hospitalier Universitaire Saint-Antoine, 27 rue Chaligny, 75571 Paris cedex 12, France; and ‡U.55 Institut National de la Santé et de la Recherche Médicale, Hôpital Saint-Antoine, 184 rue du faubourg Saint-Antoine, 75012 Paris, France

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ABSTRACT **In treating age-related osteoporosis and Paget disease of bone, it is of major importance to avoid an escape phenomenon that would reduce effectiveness of the treatment. The factors involved in the loss of therapeutic efficacy with administration of large pharmacological doses of the hormone require special consideration. Down-regulation of the hormone receptors could account for the escape phenomenon. Specific binding sites for salmon calcitonin (sCT) were characterized and localized by autoradiography on rat kidney sections incubated with ^{125}I-labeled sCT. Autoradiograms demonstrated a heterogenous distribution of ^{125}I-labeled sCT binding sites in the kidney, with high densities in both the superficial layer of the cortex and the outer medulla. Infusion of different doses of unlabeled sCT by means of Alzet minipumps for 7 days produced rapid changes in plasma calcium, phosphate, and magnesium levels, which were no longer observed after 2 or 6 days of treatment. Besides, infusion of high doses of sCT induced down-regulation of renal sCT binding sites located mainly in the medulla, where calcitonin (CT) has been shown to exert its physiological effects on water and ion reabsorption. These data suggest that the resistance to high doses of sCT often observed during long-term treatment of patients may be the consequence of not only bone-cell desensitization but also down-regulation of CT-sensitive kidney receptor sites.**

The management of disorders by calcitonin (CT) is linked to the full conservation of the biological effects of the hormone. Loss of CT activity has been observed both *in vitro*, termed the "escape phenomenon" (1), and *in vivo* during continuous administration to rats (2). Furthermore, the injection of large doses of salmon CT (sCT) in the long-term treatment of postmenopausal osteoporosis has led to a cessation of response to the hormone (3). Obviously these findings suggest that there is a loss or down-regulation of CT receptor sites during treatment with CT (3). To explain this phenomenon is of major practical importance because some therapeutic paradigms are based upon the administration of large rather than physiological doses of CT. Since not only the bone but also the kidney have been shown to be target organs for CT (4), the present study was designed to investigate whether a loss of sCT receptor sites resulting from exposure to large amounts of the hormone may be observed in the kidney as has been reported for bone (5).

The heterogeneity of kidney tissue led us to use an autoradiographic approach (6) to determine precisely the location of the possible regulation of CT binding sites. Different doses of sCT were infused subcutaneously into rats by using the Alzet minipump system. The effects of constant infusion of various concentrations of sCT on the distribution and density of sCT binding in different parts of the kidney and on plasma Ca, P_i, and Mg levels were simultaneously determined. Our data show that constant infusion of large doses of sCT induces down-regulation of renal sCT binding sites located mainly on the ascending limb of the loop of Henle, where CT has been shown to exert its physiological effects on water and electrolyte reabsorption. These results have important clinical consequences, since large doses of sCT are generally used in the treatment of osteoporosis and Paget disease of bone.

MATERIALS AND METHODS

Adult male Wistar/CF rats from our breeding colony were used. CT was infused chronically to conscious, unrestrained animals by means of osmotic minipumps.

Implantation of the Miniosmotic Pump. A small incision was made in the skin of the scapular region in rats anesthetized with ether. A small pocket was formed by spreading apart the subcutaneous connective tissues. The pumps (Alzet, model 2002), preincubated 12 hr at 37°C in isotonic saline solution (0.9% NaCl), were filled with sCT solution containing 6 M aminocaproic acid (pH 5) as peptidase inhibitor. The sCT concentration was adjusted to deliver 32 milliunits [0.5 μl (8 ng)] per hr for 24 hr, 4 days, or 7 days. Under similar conditions, a high dose [2000 milliunits (500 ng) per hr] was also infused continuously for 7 days. Each pump was inserted with the flow moderator pointing away from the incision, and the skin was closed by a wound clip. Minipumps containing only aminocaproic acid were implanted in control rats.

Hormones and Chemicals. Synthetic sCT (biological activity, 4000 international units per mg; batch 20051) was kindly supplied by Sandoz (Basel). To provide radioactive tracer, sCT was iodinated by the chloramine-T method (7) to a specific activity of 400 Ci/g (1 Ci = 37 GBq). We previously reported that ^{125}I-labeled sCT (^{125}I-sCT) retained all of the biological activity of the unlabeled sCT (6). Na^{125}I (17 Ci/ng) was obtained from Amersham. The following reagents were used: denatured human serum albumin (Centre National de Transfusion Sanguine, Paris) and the selective enzymatic inhibitor Antagosan [aprotinin; 2500 units of antiplasmin activity, corresponding to 100,000 kallikrein inhibitor units/10 ml (Hoechst, Paris La Défense, France)].

Plasma Analysis. Blood samples (average volume, 0.2 ml) were collected by retroorbital puncture in heparinized tubes and immediately centrifuged. For each experiment, rats were bled only once a day. Analyses for total plasma Ca and Mg concentrations in 20 μl of plasma were performed by atomic absorption spectrometry (Perkin–Elmer 503) and for P_i in 20 μl of plasma by the colorimetric method of Chen *et al.* (8).

Incubation and Examination of Kidney Sections. The rats were killed, and the kidneys immediately were removed and frozen in dry ice. Serial 20-μm sections were cut with a

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Abbreviations: CT, calcitonin; sCT, salmon CT.
†To whom reprint requests should be addressed.

cryostat at −18°C, mounted on gelatin-coated slides, stored overnight at −20°C, and then kept at −80°C until processed for binding studies. The slides were warmed to room temperature and incubated for 75 min in 50 mM Tris·HCl buffer (pH 7.6) containing 1% denatured human albumin and 10% Antagosan in the presence of 0.035 nM ^{125}I-sCT. Some sections were incubated with ^{125}I-sCT together with increasing concentrations of unlabeled sCT ranging from 10 pM to 0.5 μM to determine nonspecific binding. After incubation, the sections were washed four times with 40 mM Tris·HCl (pH 7.6) at 4°C for 2 min each, which gave the highest ratio between total and nonspecific binding (6). The sections were removed from the slides with Whatman GF/B filter paper and placed into plastic tubes for assay of radioactivity (counting efficiency, 60%) in a Multiprias Packard γ counter.

Some sections were incubated and washed as previously described and then dipped in distilled water and dried with cold air. Autoradiograms were prepared by a modification of the procedure described by Rostène and Mourre (9) to facilitate quantitative absorbance measurements. The sections were placed in contact with tritiated Ultrofilm (LKB) in Kodak X-Omat film holders for 12–15 days at room temperature. The films were then developed, and the densities of the enlarged autoradiographic images were measured with a densitometer that converted the amount of light into millivolts. The number of ^{125}I-sCT-binding sites was estimated by means of ^{125}I standards (9) and was expressed as fmol/mg of protein. Protein was measured by the method of Bradford (10) on scraped sections. Nonspecific binding was calculated from the amount of ^{125}I-sCT bound in the presence of excess (0.5 μM) unlabeled sCT. The results are expressed as means ± SEM. Values were compared by Student's t test, and differences were considered significant at $P < 0.05$. Scatchard analysis of the binding parameters was carried out (11), and binding constants were calculated by the least-squares method as described by Kahn et al. (12).

RESULTS

The effects of sCT infusion on plasma Ca concentrations were first tested over a period of 6 days (Fig. 1). As expected, during the first day (6 and 24 hr of infusion), plasma Ca levels significantly decreased. This effect was no longer observed after 2 days of treatment with 32 milliunits per hr. With 2000 milliunits per hr, the hypocalcemic response was not enhanced after 6 hr of infusion but was significantly higher after 1 day and persisted for at least 4 days. In both cases, plasma Ca levels returned to control values by 6 days of infusion.

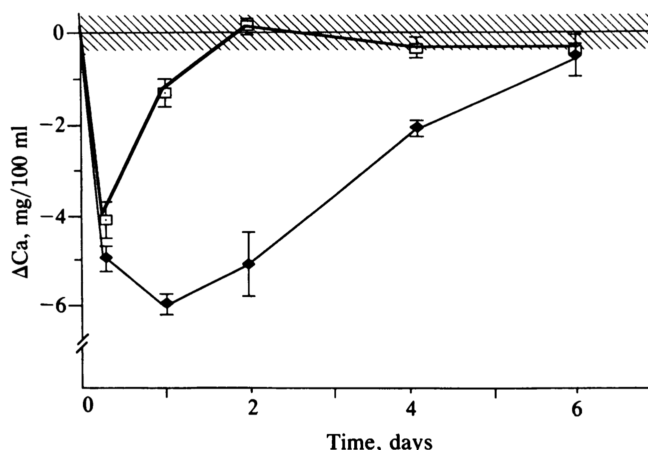

FIG. 1. Effects of sCT infusion on plasma concentration expressed as the difference (ΔCa) between control and treated rats. Mean value for control plasma Ca is 11.8 ± 1.53 mg/dl; five rats were used per group. The hatched area shows the upper and lower limits of a significant change in plasma levels of control rats ($P < 0.001$). □, Infusion of 32 milliunits per hr; ◆, infusion of 2000 milliunits per hr.

Infusion of 32 milliunits of sCT per hr also decreased P_i and enhanced Mg plasma levels during the first day (Table 1). This effect, as with Ca, was no longer observed after several days of infusion.

To determine if this loss of effect after several days of infusion was due to a loss of biological activity of the hormone, the same minipumps containing 32 milliunits per hr were transplanted again to age-matched control rats. The significant decrease in plasma Ca concentrations (ΔCa = −2.2 ± 0.2 mg/dl; $P < 0.001$) observed 6 hr later and the significant hypophosphatemic ($\Delta P_i = -2.8 \pm 0.2$ mg/dl) and Mg effects (+0.29 ± 0.05 mg/dl) clearly demonstrated that sCT was still biologically active in our experimental conditions.

Binding of ^{125}I-sCT on Rat Kidney Sections. Optimal binding conditions of ^{125}I-sCT on rat kidney sections as previously determined (6) demonstrated that specific binding reached equilibrium after 1 hr of incubation and that there is only a single class of binding sites for ^{125}I-sCT. The number of ^{125}I-sCT binding sites decreased by 15% after 7 days of infusion with 32 milliunits per hr (Fig. 2). However, the infusion of 2000 milliunits per hr for 7 days caused an 80% decrease in the number of binding sites, with no modification of the dissociation constant with either dose of sCT (Table 2). A similar decrease in the number of ^{125}I-sCT binding sites was obtained with kidney sections from rats infused for 7 days with the lower dose of sCT (32 milliunits per hr) and killed 24 hr after removal of the pumps (control, 244 ± 10 fmol/mg of protein; treated, 188 ± 7 fmol/mg of protein).

Autoradiographic Studies. ^{125}I-sCT binding sites in autoradiograms of kidney sections from control rats were mainly concentrated in the outer medulla (Fig. 3 and Table 3). A high density of ^{125}I-sCT was observed in both the inner and the outer zone of the outer medulla, whereas a moderate labeling was seen in the medial part of the outer medulla. Moreover, quite intense patchy labeling was also found in the cortex and was concentrated mainly in the superficial zone (Fig. 3A and Table 3).

In the presence of an excess (0.5 μM) of unlabeled sCT, the labeling almost disappeared from all levels (Fig. 3B).

Autoradiograms of rat kidney sections from rats infused with 32 milliunits of sCT per hr showed that the decrease of ^{125}I-sCT binding was localized in both the outer medulla and the cortex (Fig. 3C and Table 3). More dramatic effects occurred with infusion of 2000 milliunits of sCT per hr for 7 days, producing a major decrease in the density of ^{125}I-sCT binding sites (Fig. 3D and Table 3). Although this dose affected all parts of the kidney, the greatest decrease was mainly observed in the outer medulla (Fig. 3D).

Table 1. Effects of constant infusion of sCT at 32 milliunits per hr on plasma P_i and Mg

Days of infusion	ΔP_i,* mg/dl ± SEM	ΔMg,† mg/dl ± SEM
0.25	−3.01 ± 0.23‡	0.15 ± 0.06‡
1	−1.96 ± 0.28‡	0.29 ± 0.05‡
4	−0.86 ± 0.19‡	−0.09 ± 0.03§
6	−0.34 ± 0.15¶	−0.04 ± 0.02¶

*Mean control value = 8.20 ± 0.25 mg/dl.
†Mean control value = 1.61 ± 0.02 mg/dl.
‡$P < 0.001$ vs. respective control values in nonimplanted rats.
§$P < 0.005$ vs. respective control values in nonimplanted rats.
¶Not significant.

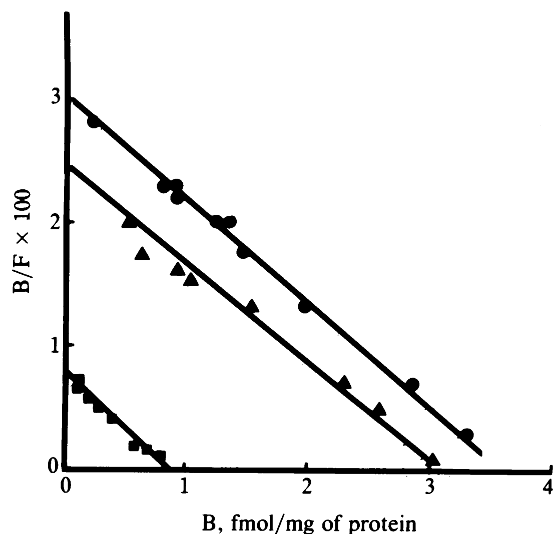

FIG. 2. Scatchard analysis of ^{125}I-sCT binding sites in rat kidney sections. ●, Controls; ▲, after 7 days of continuous infusion of sCT at 32 milliunits per hr; ■, after 7 days of continuous infusion of sCT at 2000 milliunits per hr. Bound-to-free ratio of ^{125}I-sCT (B/F) was plotted as a function of the quantity of CT bound (B) to the receptor. Regression lines were used for computation by the least-squares method (12) for the determination of the dissociation constant and the number of binding sites (Table 2).

DISCUSSION

The present data demonstrate that continuous administration of sCT *in vivo* induces down-regulation of renal sCT binding sites, as evidenced by quantitative autoradiography. Scatchard analyses indicated that this effect was due to a reduction of ^{125}I-sCT binding sites with no change in the apparent affinity for the peptide. The assessment that the peptide was not degraded in the minipumps under our experimental conditions and that it was still able to induce changes in ion concentrations after 7 days in the pumps shows that the effect observed on the kidney is not due to a loss of sCT activity or possible metabolites of the peptide. A similar observation was reported by Tashjian *et al.* (5), who showed a decrease in CT binding sites in cultured bone cells in the presence of CT. Those authors suggested that the loss of available sites was due to the occupancy of the receptor sites by a poorly dissociable hormone. Even if we cannot exclude the possibility that a small fraction of unlabeled sCT may occupy the binding sites, our data suggest that down-regulation is probably not due to occupation of sites for the following reasons. (*i*) A similar decrease (see *Results*) and pattern of distribution of binding on the autoradiograms (not shown) was observed in rats killed 24 hr after removal of the pump and in animals in which the pump was still present at the time of sacrifice. (*ii*) Treatment of kidney sections from either control or perfused (2000 milliunits per hr) rats with glycine·HCl (pH 2.6) (13) resulted in a 90% loss of specific binding, suggesting that washing procedures of the sections gave similar data

Table 2. ^{125}I-sCT binding parameters in control and infused rats

Rats	^{125}I-sCT binding parameters ± SEM: K_d, M × 10^9	No of sites, fmol/mg of protein
Control	1.5 ± 0.2	220 ± 22
Infused		
32 milliunits/hr	1.6 ± 0.1	185 ± 5
2000 milliunits/hr	1.2 ± 0.1	40 ± 4*†

Data from three different experiments were computed using Scatchard analysis of displacement curves resulting from exposure of rat kidney sections to increasing concentrations from 10 pM to 0.5 μM of unlabeled sCT.
*$P < 0.01$ vs. control rats.
†$P < 0.01$ vs. infused rats (32 milliunits per hr).

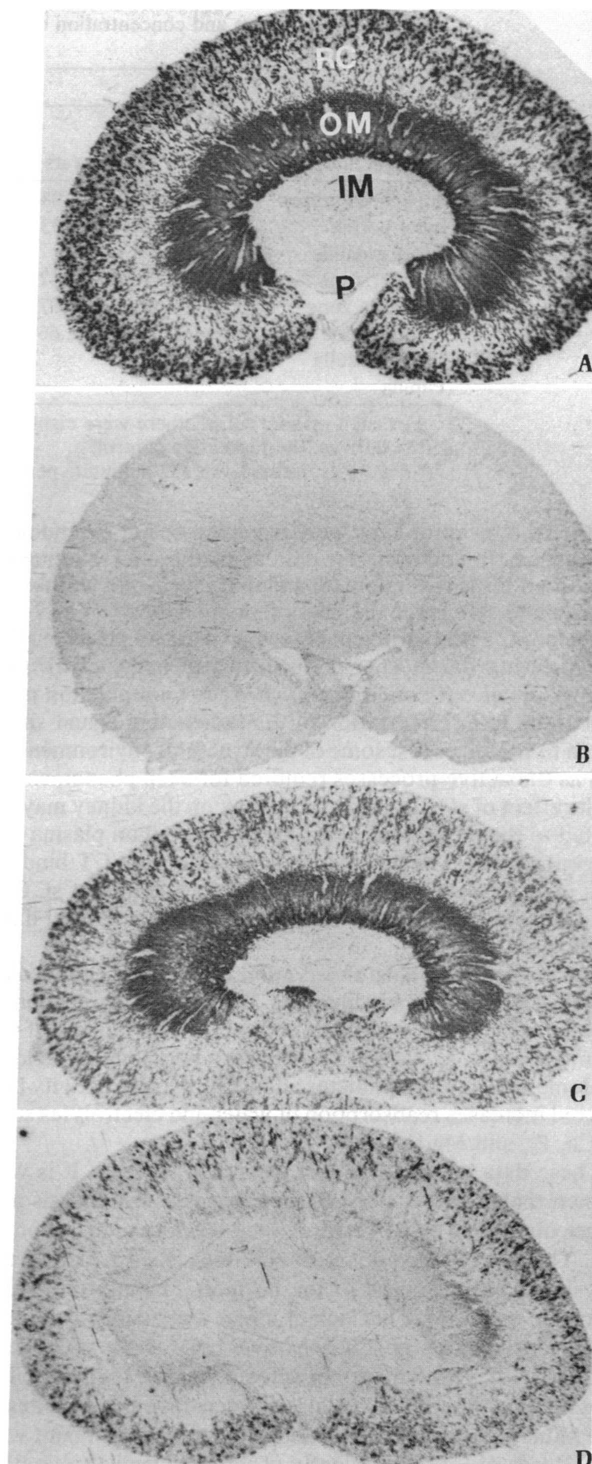

FIG. 3. Autoradiograms of 20-μm sections of rat kidneys. RC, renal cortex; OM, outer medulla; IM, inner medulla; P, papilla. (*A*) Section incubated with 0.035 nM ^{125}I-sCT alone. (*B*) Section incubated with 0.035 nM ^{125}I-sCT in the presence of 0.5 μM unlabeled sCT. (*C*) Section from a rat infused 7 days with unlabeled sCT at 32 milliunits per hr. (*D*) Section from a rat infused 7 days with unlabeled sCT at 2000 milliunits per hr. Binding conditions and procedures are described in the text. Densitometric values obtained from autoradiograms are reported in Table 3.

Table 3. Localization and concentration of ^{125}I-sCT binding sites in kidney sections determined by densitometric analysis

	^{125}I-sCT binding sites, fmol/mg of protein		
		Continuous sCT infusion for 7 days	
Structures	Control rats	32 milliunits/hr	2000 milliunits/hr
Superficial cortex	44.60 ± 2.98	11.19 ± 0.80*	6.77 + 0.84*†
Inner cortex	5.46 ± 0.33	1.64 ± 0.48*	–*†
Outer medulla			
Outer zone	26.37 ± 1.57	19.64 ± 1.04*	2.30 ± 0.38*†
Middle zone	11.66 ± 1.07	11.21 ± 1.30	–*†
Inner zone	23.79 ± 2.66	26.00 ± 1.75	–*†
Inner medulla	–	–	–
Papilla	–	–	–

For each mean ± SEM, there were eight determinations; –, less than background.
*$P < 0.01$ vs. the respective control.
†$P < 0.01$ vs. infused rats (32 milliunits per hr).

whether the receptor sites were occupied or not by endogenous ligand. In addition, our data show that sCT binding in our conditions is reversible (6) and that four 2-min washes of the sections after the incubation with either ^{125}I-sCT or unlabeled sCT was sufficient enough to wipe off possible sCT bound during the *in vivo* perfusion (data not shown). (*iii*) Finally, under our conditions *in vitro*, down-regulation may not be due to internalization of the radioactive ligand since frozen tissue slices lost some of the structural environment as well as the active processes required for such phenomenon.

The effect of sCT infusion for 1 week on the kidney may be related to the physiological changes observed on plasma ion concentrations. The loss or desensitization of sCT binding sites in the kidney may be related to the inability of sCT to induce changes in plasma Ca, P_i, and Mg after several days of infusion.

Our autoradiographic observation showing that the major decrease in ^{125}I-sCT binding sites following sCT infusion is located in the outer medulla is consistent with binding of the hormone at the level of the thick ascending limb of the loop of Henle, where CT stimulates adenylate cyclase activity (14, 15) and increases reabsorption of water and electrolytes such as Ca, P_i, and Mg (16–18).

These data may be relevant to human therapy. It is well known that treatment of postmenopausal osteoporosis and Paget disease of bone requires long-term administration of CT. The use of large doses of sCT may elicit a loss of the physiological responses to the hormone, mainly the hypocalcemic effect (3). This lack of effect was mainly attributed to desensitization of CT-sensitive bone cells of pagetic patients (19). Our data are in agreement with the possibility that not only bone cells but also CT-sensitive binding sites in the kidney are desensitized under long-term treatment with high doses of CT. We recently observed a similar alteration in CT receptor sensitivity in the kidney of Wag/Rij rats bearing a transplanted medullary thyroid carcinoma and characterized by an abnormal secretion of CT (20). Thus, clinical investigation of the kidney function has to be taken into account in the long-term treatment of patients with high doses of CT.

Down-regulation appears to be a dose-dependent phenomenon. Large doses of sCT decreased the number of sites by 80%. These results suggest that the resistance to high doses of sCT during long-term treatment of patients may not be the result of the production of antibodies (21, 22) but may involve down-regulation of CT-sensitive kidney and bone receptor sites.

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