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Improvement and Initial *In Vivo* Application of the Radioimmunoassay of Rat Thyrocalcitonin[1, 2] (39170)

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(Introduced by Roy V. Talmage)

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Recently our laboratory reported the development of the first homologous radioimmunoasay for rat thyrocalcitonin (TCT) using highly purified native rat TCT and guinea pig antisera to rat TCT (1). Because rat TCT closely resembles human TCT in its amino acid composition (1), we found, in agreement with Milhaud *et al.* (2), that purified human TCT and antisera to human TCT also could be used to measure rat TCT in a heterologous assay system (1). Evaluation of rat thyroid gland extracts further showed that our results obtained by radioimmunoassay of rat TCT accurately reflected those obtained independently by bioassay (1).

A major problem with our initial immunoassay for rat TCT was that, while our antisera to human or rat TCT could easily measure the rat hormone in thyroid extracts or thyroid venous blood, the lower limits of detectability of these antisera (2–3 ng/ml serum) were too high to detect rat TCT reliably in peripheral blood even during hypercalcemia (1). In the present study we have developed antisera to rat TCT in the chicken which are approximately one order of magnitude more sensitive (0.1–0.2 ng/ml serum) than our earlier guinea pig antisera. These new antisera have permitted us to begin to evaluate changes in TCT in rat peripheral blood. This report describes our initial studies designed to evaluate changes in circulating immunoreactive TCT in the rat.

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Materials and methods. Animals. Male Holtzman rats (Holtzman Co., Madison, Wis.), 30–40 days old and weighing 120–200 g were used. For each experiment, all rats had been born on the same day and were received in the same shipment. Except where noted in the legends, all rats were allowed food (Purina Laboratory Chow) and water *ad libitum*.

Treatments. Hot wire cautery of the lower pole of each lobe of the thyroid gland was performed as previously described (3). Calcium was administered either iv or orally (by gavage) using 0.1 *M* $CaCl_2$. Administration iv was by injection (0.8 ml/100 g body wt) via a lateral tail vein. Gavage was accomplished by delivery of the desired volume via an 18-gauge metal feeding tube.

Other test agents were *d*,l-isoproterenol hydrochloride (donated by Winthrop Labs, New York, N.Y.) and porcine Gastrin I and II (Wilson Labs, Chicago, Ill.). Isoproterenol was dissolved and diluted in 0.15 *M* NaCl; gastrins were dissolved in a small volume of 0.1 *N* ammonium hydroxide and then diluted in 0.15 *M* NaCl. Control rats were either left untreated or received an appropriate volume of NaCl iv. Thyroidectomy (to obtain TCT-free serum) or sham operations (exposure of the thyroid gland) were achieved by blunt dissection under ether anesthesia.

Blood collection and analysis. Blood samples were obtained by centrifugation, and the calcium concentration was determined within 2 hr of blood collection by the automated (AutoAnalyzer) method of Gitelman (4). The remaining portion of each serum sample was stored at −20° for subsequent immunoassay. For radioimmunoassay, all serum samples from the same experiment were analyzed in the same assay.

Radioimmunoassay of rat TCT. Details of the procedure used for radioimmunoas-

say of rat TCT have been described recently in detail (1). With this procedure intra- and interassay variations, as reported previously (1), did not exceed 10 and 20% respectively. Highly purified native rat TCT was used for iodination with ^{125}I and as unlabeled reference standard. Chicken antisera to rat TCT were developed by injecting young laying hens with partially purified rat TCT. For immunization, TCT solution was administered im (breast muscle) emulsified in an equal volume of complete Freund's adjuvant. Volume injected ranged from 0.5 to 4 ml, but no more than 1 ml was given at a single injection site. Initial immunizations were with 50 MRC U TCT/bird and subsequent injections at 2-week intervals were with 30 MRC U/bird. At each 2-week interval after the third injection, 5 to 20 ml of blood was drawn from a wing vein or from the heart, the bird being allowed to survive. Each serum sample was evaluated routinely for titer and then, using reference standard, for its sensitivity (i.e., lower limit of detectability). Antisera from two chickens (of four immunized to date) exhibited adequate titers and sensitivity (Fig. 1) and were used for these studies.

Incubations were conducted as previously described (1). However, "nonequilibrium" conditions were employed, reference standard or unknown sera being incubated with antiserum for 2 days in the absence of labeled TCT and for an additional 2 days after addition of labeled hormone. The two chicken antisera were used at final

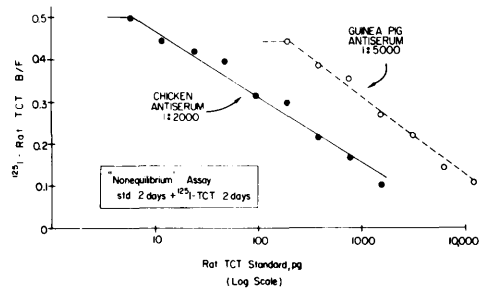

FIG. 1. Comparison of standard curves obtained in the same assay using either a guinea pig antiserum (final dilution 1:5000) or a chicken antiserum (final dilution 1:2000) to rat TCT. B/F = bound to free ratio of labeled rat TCT. Incubation was for 2 days in the absence of labeled hormone and for an additional 2 days after addition of labeled hormone.

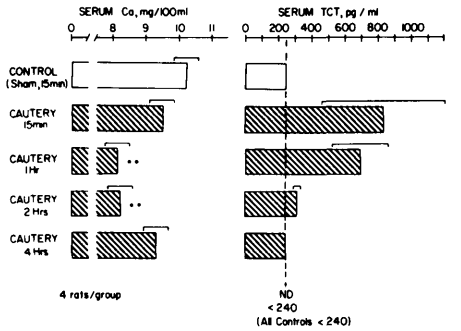

FIG. 2. Changes in TCT and calcium in serum of rats at various times after cautery of the thyroid gland. Control rats were sham-operated and bled 15 min later. Each bar represents the mean serum calcium or TCT for a separate group of four rats, and the brackets show the SE. TCT for all control rats was undetectable (<240 pg/ml), the dashed line showing the lower limit of detectability of the immunoassay. For serum Ca in this and subsequent figures, P values vs control: ** = $P < 0.01$, *** = $P < 0.001$. For serum TCT in this and subsequent figures, each bar above the dashed line differs from the control bar by $P \leqq 0.05$.

dilutions of 1:2000 and 1:10,000, respectively. Incubations were in a final volume of 0.5 ml. Unknown sera were tested in final concentrations of 10% (v/v) or less, and all tubes were adjusted to a final serum concentration of 10% using thyroidectomized rat serum obtained from rats thyroparathyroidectomized for 24 hr or longer. Unknown sera also were incubated in the absence of antiserum to allow correction for nonspecific effects (5). After incubation, bound and free labeled hormone were separated using dextran-coated charcoal, and both fractions were counted by solid scintillation spectrometry.

Statistical analysis. Data on serum calcium were subjected to analysis of variance; standard errors were calculated from the residual error term, and the significance of differences between mean values was assessed by the F test and the multiple comparisons test of Hartley (6). For TCT, standard errors tended to be large but, nevertheless, in groups where TCT was detectable, each rat exhibited a measurable serum level; conversely, in control groups, there was no measurable serum level of TCT in any rat. For TCT, the significance of differences between control and treated groups

was assessed using both χ^2 and the nonparametric test of Wilcoxon for unpaired measurements (6). Using these tests, in all groups of rats where TCT was detectable, these groups differed from the control group by at least $P < 0.05$.

Results. Figure 1 shows a representative standard radioimmunoassay curve obtained with one of our chicken antisera to rat TCT. Both chicken antisera used proved capable of detecting as little as 6–12 pg of rat TCT reference standard. Since unknown sera were tested in final concentrations not exceeding 10%, the lower limit of detectability in the present study was 120–240 pg/ml for unknown serum samples. In Fig. 1 the standard curve obtained with one of the newer chicken antisera also is compared to that obtained in the same assay with one of our earlier guinea pig antisera. The results show clearly that the chicken antiserum is approximately one order of magnitude more sensitive than the guinea pig antiserum.

Table I shows results of our initial attempt to measure rat TCT in peripheral blood. In this and the other experiments to be shown, TCT levels in control rats could not be measured, being less than 120–240 pg/ml in all cases. However, as shown in Table I, 5 min after provocative challenge with iv calcium or by thyroid cautery, TCT in serum rose by at least 6- to 9-fold to levels of ~1000–2000 pg/ml. Figure 2 shows additional results obtained at various times after thyroid cautery. TCT levels again were high shortly after cautery (15 min) and before serum calcium had begun to fall noticeably. The maximum hypocalcemic effect after cautery was observed at 1–2 hr, during which time TCT in serum returned almost to undetectable levels. By 4 hr after cautery, serum calcium had returned to normal, and TCT in serum was no longer detectable.

Figure 3 shows additional results obtained at two times intervals after iv injection of calcium. The TCT secretory response appeared to follow the pattern of change in serum calcium. TCT levels were highest at 5 min after injection when serum calcium was highest. Since TCT in control rats was <120 pg/ml, the serum TCT rose at least 20-fold by 5 min. By 30 min, serum calcium had returned toward normal, and the TCT level had fallen perceptibly. In this experiment administration of the β-adrenergic agonist drug, isoproterenol, also raised serum TCT into the detectable range.

Figure 4 shows results obtained after administration of calcium orally by gavage. Here serum calcium was not significantly altered 30 or 60 min later by the smaller dose of calcium administered (3.7 mg/100 g body wt), and TCT levels were not detectable. However, administration of a larger

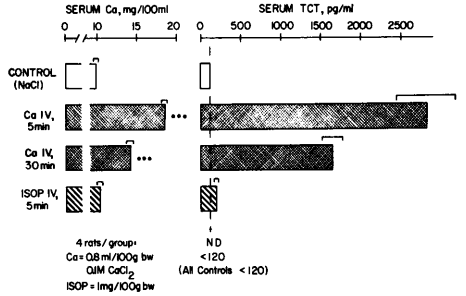

FIG. 3. Changes in TCT and calcium in serum of rats at 5 and 30 min after iv injection of calcium and 5 min after iv injection of isoproterenol (ISOP). Control rats received 0.15 *M* NaCl iv (0.8 ml/100 g) and were bled 5 min later. Each bar represents the mean serum calcium or TCT for a separate group of four rats, and the brackets show the SE. TCT for all control rats was undetectable (<120 pg/ml).

TABLE I. CHANGES IN TCT AND CALCIUM IN SERUM OF RATS 5 MIN AFTER EITHER IV INJECTION OF CALCIUM OR CAUTERY OF THE THYROID GLAND.[a]

Treatment	Serum concentration	
	Calcium (mg/100 ml)	TCT (pg/ml)
A. None (control)	10.7 ± 0.19	N.D. (<240)
B. 0.1 *M* $CaCl_2$ iv	16.3 ± 0.19***	2220 ± 337*
C. Thyroid cautery	9.7 ± 0.19**	1425 ± 460*

[a] N.D. = not detectable (all control values below limits of detectability, i.e., <240 pg/ml). Each value represents the mean ± SE for a separate group of four rats, and differs from control group: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

dose of calcium (12.2 mg/100 g) produced a modest increase in serum calcium at 30 and 60 min and increased serum TCT to approximately 500 pg/ml.

Table II shows results of experiments designed to evaluate whether or not increased TCT levels could be detected in the rat after acute administration of gastrin or its active analog, pentagastrin. Despite the fact that large doses of gastrin were tested, detectable levels of TCT in serum were not observed at 5 (Expts 1 and 2) or 30 min (Expt 3) after injection. The fact that in these same experiments hypercalcemia increased serum TCT (Expts 2 and 3) and hypocalcemia, as previously reported (7), was observed 30 min after gastrin injection (Expt 3) served to assure that the rats used in these experiments were not completely unresponsive.

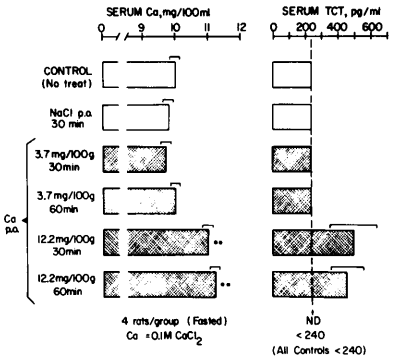

FIG. 4. Changes in TCT and calcium in serum of rats at 30 and 60 min after intragastric administration of two different doses of calcium by gavage (p.o.). Control rats were untreated; an additonal group was given 0.15 *M* NaCl (5 ml) by gavage and bled 30 min later. Other groups received 0.1 *M* $CaCl_2$, either ~1.5 ml or ~5 ml/rat (to give 3.7 or 12.2 mg Ca/100 g) and were bled 30 or 60 min later. Each bar represents the mean serum calcium or TCT for a separate group of four rats, and the brackets show the SE. TCT for all control rats and all rats receiving 3.7 mg Ca/100 g was undetectable (<240 pg/ml). Rats were fasted for 18 hr before the experiment.

Discussion. The results of this study show that antisera to rat TCT developed in the chicken can be applied to the measurement of elevated levels of TCT in the peripheral blood of the rat. These antisera have proved to be more sensitive (~0.1 to 0.2 ng/ml serum) in terms of their ability to detect rat TCT than our previous antisera raised in the guinea pig (Fig. 1). Even using the chicken antisera, basal levels of immunoreactive TCT in young male Holtzman rats were undetectable, being less than 100–200 pg/ml, as has been reported for other species including man (8–10). However, the present assay system could be used to define TCT concentrations in peripheral rat serum quantitatively after provocative challenge.

Not surprisingly, iv calcium in an amount sufficient to induce severe hypercalcemia produced rapid and large increases in serum TCT (Tables I, II; Fig. 3). Cautery of

TABLE II. CHANGES IN TCT AND CALCIUM IN SERUM OF RATS 5 OR 30 MINUTES AFTER IV INJECTION OF A LARGE DOSE OF PENTAGASTRIN OR PORCINE GASTRIN.[a]

Expt no. (time, blood collection)	Treatment	Serum concentration	
		Calcium (mg/100 ml)	TCT (pg/ml)
1 (5 min)	A. Control (NaCl iv)	9.9 ± 0.24	N.D. (<120)
	B. Pentagastrin (25 μg/rat iv)	10.1 ± 0.24	N.D. (<120)
2 (5 min)	A. Control (NaCl iv)	10.5 ± 0.26	N.D. (<240)
	B. Porcine gastrin (50 μg/rat iv)	10.1 ± 0.26	N.D. (<240)
	C. 0.1 *M* $CaCl_2$ iv	18.7 ± 0.26***	3508 ± 432**
3 (30 min)	A. Control	10.0 ± 0.18	N.D. (<240)
	B. Porcine gastrin (37 μg/rat iv)	9.4 ± 0.18*	N.D. (<240)
	C. 0.1 *M* $CaCl_2$ iv	12.2 ± 0.15***	1086 ± 297**

[a] N.D. = not detectable (all control values = <120 or <240 pg/ml). Each value represents the mean ± SE for a separate group of four to five rats. In Expt 3, rats were fasted for the 18-hr period before the experiment. Values differ from control group: * $P < 0.05$; ** $P \leq 0.01$; *** $P < 0.001$.

the thyroid gland also rapidly induced marked increases in circulating TCT (Table I, Fig. 2), thus demonstrating that the original hypothesis of Hirsch *et al.* (3) that thyroid cautery releases TCT in the rat was indeed correct. In sufficient amounts, oral calcium also was shown to elevate serum TCT (Fig. 4) as originally inferred but not shown directly by Gray and Munson (11).

In these experiments we were not able to detect TCT in rat serum after injection of large doses of pentagastrin or gastrin (Table II). This is in contrast to our findings in the pig (12, 13) and in patients with medullary thyroid cancer (9) where gastrin and pentagastrin can produce TCT secretory responses equivalent to or even exceeding those produced by iv calcium. It is possible in the present study that TCT levels increased in response to gastrin but did not exceed the limits of detection of the immunoassay and thus went unnoticed. Alternatively, it is possible that gastrin is not an effective secretagogue for TCT in the rat. Although gastrin can produce modest hypocalcemia in the rat (7, 14; Table II), it has been reported to do this in the absence of the thyroid gland (14). Furthermore, the doses of gastrin required to produce hypocalcemia in the rat are exceedingly high, the amount required for a single 150-g rat (e.g., 20–40 μg) being sufficient to evoke a maximal TCT secretory response in a 20-kg pig (12, 13). This fact, together with the reported high doses of gastrin required to evoke gastric acid secretion in the rat (15, 16), suggests that gastrin may not be as potent a TCT secretagogue in the rat as it has proved to be in certain other mammalian species.

Interestingly, as shown in Fig. 3, β-adrenergic amines such as isoproterenol can elevate blood levels of TCT in the rat. This finding is in agreement with our earlier report that these catecholamines promote TCT secretion and prevent, in thyroid-intact rats, the marked hypercalcemia observed after catecholamine injection into acutely thyroidectomized rats (17).

Detection of basal TCT levels in peripheral blood of young Holtzman rats must await either development of even more sensitive antisera than those used here or improvements in existing assay conditions. Nevertheless, the present assay system is suitable for evaluating elevated circulating levels of TCT in these young Holtzman rats. In other preliminary studies, we have detected increases in TCT levels after feeding in rats adapted to a regular standardized schedule of daily feeding (18). Still other studies currently in progress (Peng and Cooper, unpublished results) suggest that basal levels of TCT may be measured by the present radioimmunoassay in older Holtzman rats, in rats from certain suppliers other than the Holtzman Co., and in goitrous or chronically hypocalcemic rats all of which possess thyroidal TCT levels higher than in the young Holtzman rats used for this study (reviewed in (19)). Such animals may serve to define and quantitate changes which occur in TCT secretion in the rat more adequately and, thus, help to assess the physiological role of TCT in this species.

Summary. Previously we reported a homologous radioimmunoassay for rat thyrocalcitonin (TCT) which was sensitive enough (2–3 ng/ml serum) to measure TCT in thyroid venous blood or thyroid gland extracts but could not detect TCT in peripheral blood even after provocative challenge with iv calcium. In the present study chicken antisera to rat TCT were developed which were sufficiently sensitive (120–240 pg/ml serum) to permit initial evaluation of changes in TCT in rat peripheral blood. The following results were observed: (1) Basal serum TCT in young male Holtzman rats was undetectable, being <120–240 pg/ml; (2) induction of marked hypercalcemia by iv calcium increased TCT to ~1000–3000 pg/ml within 5 min; (3) thyroid cautery increased TCT to ~1000 pg/ml in 5–15 min; (4) calcium gavage (12.2 mg Ca/100 g) produced modest hypercalcemia in 30–60 min and increased serum TCT to ~500 pg/ml; (5) injection of isoproterenol raised serum TCT detectably; (6) injection of large doses of gastrin or pentagastrin did not produce detectable increases in TCT 5 or 30 min later.

The results show that suitable antisera to rat TCT can be developed in chickens and applied to the measurement, by radioimmunoassay, of elevated circulating levels of TCT in the rat.

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