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Concurrent Secretion of Calcitonin and Parathyroid Hormone *in Vitro* from the Rat Thyroparathyroid Complex[1,2] (40192)

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In most mammalian species the thyroid and parathyroid glands reside in close anatomical association. In the rat, normally only two parathyroid glands are present, and, typically, they are located one on each lateral lobe of the thyroid gland (1).

After our laboratory developed the first homologous radioimmunoassay for measurement of calcitonin (CT) in the rat (2, 3), we utilized the assay to study secretion of CT *in vitro* from baby rat thyroid glands incubated in a chemically defined medium (4). In these experiments no attempt was made to remove the parathyroid glands, since they did not interfere with the study of CT release and since we felt their removal might damage the thyroid tissue and produce nonspecific release of CT.

This paper reports our application of a radioimmunoassay for bovine parathyroid hormone (PTH) to the measurement of rat PTH. With assays capable of measuring both CT and PTH in the rat, we have examined effects of calcium and other test agents on concurrent secretion of CT and PTH from rat thyroparathyroid gland complex *in vitro*.

Materials and methods. Incubation procedure. Details of the incubation procedure and the chemically defined medium employed have been reported previously (4). In brief, thyroparathyroid gland complexes were removed from 8-day-old Zivic-Miller rat pups and incubated in 2 ml serum-free medium for up to 8 hr (one gland complex/flask). Flasks were incubated with constant shaking at 37° under an atmosphere of 95% O_2–5% CO_2. Individual experiments typically consisted of 36–40 flasks in treatment groups of 6–12 flasks each. Samples of medium (0.3 ml) were removed after 4 hr of incubation. Medium samples at 4 and 8 hr and tissue samples all were stored at −20° until analyzed.

Test agents. Synthetic substance P was purchased from Beckman (Palo Alto, CA). Neurotensin was kindly donated by Dr. Susan Leeman, Harvard Medical School. Pentagastrin was supplied through the generosity of Ayerst Labs (New York, NY). Secretin was purchased from Sigma (St. Louis, MO). The freshly weighed powders were dissolved directly in the incubation medium.

Synthetic analogues of vitamin D_3 were dissolved in 95% ethanol and added to culture medium at dilutions of 1:150 or greater. Appropriate vols of ethanol were added to control flasks. The 1,25-$(OH)_2D_3$ was donated by Hoffman-LaRoche (Nutley, NJ) and the 24,25-$(OH)_2D_3$ was supplied by Dr. Anthony Norman (Univ. of California, Riverside).

Analyses. Calcium concentrations in aliquots of freshly prepared media were determined by atomic absorption spectrophotometry to verify calculated concentrations. Samples of medium were analyzed for CT using a radioimmunoassay for rat CT described previously in detail (3, 4). The assay was performed under nonequilibrium conditions using a chicken antiserum to native rat CT and highly purified native rat CT as reference standard and iodinated tracer. The phase separation was achieved with dextran-coated charcoal, and both bound and free labeled hormone were counted.

Samples of medium were analyzed for PTH using a bovine PTH radioimmunoassay

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system. Approximately 20 guinea pig and rabbit antisera to bovine PTH were assessed for their ability to crossreact with rat PTH. This was done by assaying a crude homogenate of adult rat parathyroid glands (5 glands/ml in 0.15 *M* NaCl). As a control tissue, rat skeletal muscle was homogenized to yield an homogenate of equivalent protein concentration, i.e. ~200 μg/ml (5). Analysis of both homogenates revealed that four different guinea pig antisera reacted with the rat parathyroid homogenate (440 ± 105 ng PTH/ml in terms of 1–84 bovine PTH reference standard) but not with the muscle homogenate (<0.490 ng/ml). The calculated concentration of PTH was 27.5 ± 6.6 ng/gland. The parathyroid homogenate (but not the muscle homogenate) also reacted with three separate antisera to synthetic human 1–34 PTH, but the values obtained in terms of 1–34 reference standard were consistently much lower (<1.0 ng/ml), suggesting that antisera to 1–34 human PTH would not be very useful for measuring 1–84 rat PTH. One of the guinea pig antisera (supplied by Dr. Louis Sherwood, Michael Reese Hospital) was selected and used for all the incubation studies at a final dilution of 1:40,000. Highly purified bovine 1–84 PTH, kindly supplied by Dr. Henry Keutmann, Mass. General Hosp., was used as unlabeled reference standard and as iodinated tracer.

Assays were conducted under nonequilibrium conditions, labeled hormone being added for 2–3 days after an initial sample incubation of 2–3 days. A representative standard curve is shown in Fig. 1. All samples from a single experiment were included in the same assay, and samples were thawed only one time, assays for both CT and PTH being conducted concurrently. In initial assays, multiple dilutions of media produced immunoassay curves indistinguishable from those produced by PTH reference standard. Thereafter, samples were assayed in triplicate in volumes of 50–100 μl. Incubation buffer for PTH assays was 0.02 *M* sodium barbital (pH 8.6) containing thimerosal (1 mg/10 ml) and Trasylol (500 KIU/ml). Phase separation was by "second antibody" precipitation using rabbit anti-guinea pig gamma globulin serum, and both bound and free labeled hormone were counted by crystal scintillation spectrometry.

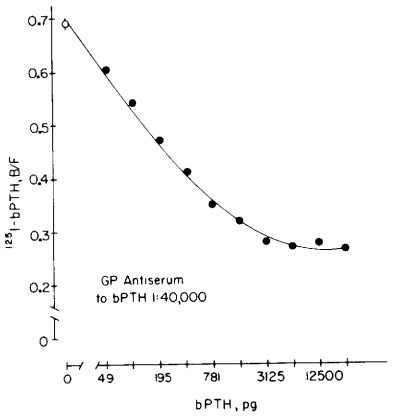

FIG. 1. Representative standard curve obtained with the radioimmunoassay for bovine PTH used to measure rat PTH in incubation media and rat parathyroid gland homogenates. Reference standard was 1–84 bPTH. Multiple aliquots of gland extracts and incubation medium produced changes in B/F ^{125}I-bPTH indistinguishable from those produced by reference standard (see Methods).

Statistical analyses. Assays were subjected to computer analysis. Mean values ± SEM were calculated for each treatment group if all values exceeded detection limits of the radioimmunoassay; significance of differences between mean values was determined by an *F* test (6). If some or all values in a treatment group were below the detection limits of the assays for CT or PTH, the significance of differences between groups was evaluated using the nonparametric test of Wilcoxon (6).

Results. Previously we reported that CT levels are easily measured (~40 ng/gland) in individual 8-day-old, baby rat thyroparathyroid complexes incubated in 1 m*M* Ca for 8 hr (4). In the present study it was not possible to measure PTH in individual gland complexes whether they had been incubated or not. Even when groups of six complexes were pooled and homogenized in a 1 ml vol, the amount of PTH was too small to be detected (<0.8 ng/gland) unless the glands had been incubated 8 hr in 2.5 m*M* Ca which suppressed PTH release, apparently allowing hormone to accumulate (see below). In the latter case a mean level of 3.8 ng PTH/gland complex was detected.

Effects of medium [Ca]. As shown in Figs. 2 and 3, secretion of PTH and CT were altered markedly by changes in the medium

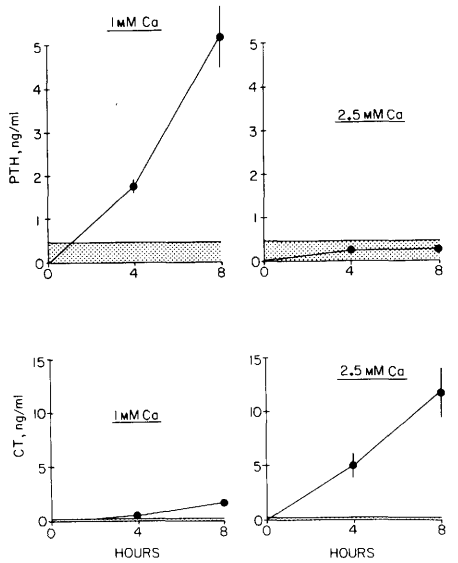

FIG. 2. Concurrent changes in immunoreactive CT and PTH released into medium during incubation of rat thyroparathyroid glands complexes. In two groups of 12 flasks each, thyroparathyroid glands (1 per flask in 2 ml) were incubated in either 1 m*M* Ca or 2.5 m*M* Ca. Upper and lower graphs show levels of PTH and CT, respectively, in the same samples. Each point represents the mean of 12 values and the vertical lines show the SE. If no vertical line is shown, SE was too small to depict. Shaded areas indicate the lower limits of detectability of the immunoassays for PTH and CT.

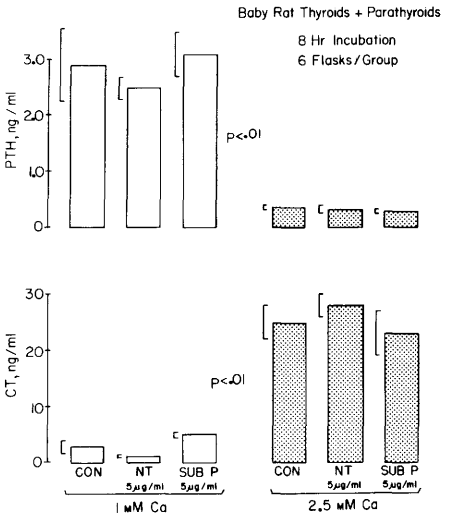

FIG. 3. Lack of effect of substance P (SUB P) and neurotensin (NT) on concurrent release of immunoreactive CT and PTH from rat thyroparathyroid glands *in vitro*. Flasks containing either control medium (CON) or medium containing test agents were incubated for 8 hr. Each bar represents six flasks and the brackets show the SE. *P* values refer to comparisons of flasks incubated in 1 m*M* Ca vs 2.5 m*M* Ca. Samples of medium containing test agents but not incubated with tissues ("0 time" samples), were included in immunoassays to test for possible nonspecific effects, but none were found in this or other experiments.

Ca concentration. As we had found previously, CT release was low at 1 m*M* Ca and increased five- to tenfold when glands were incubated in 2.5 m*M* Ca for 8 hr (Fig. 2). At 1 mM Ca, PTH secretion was easily measured and increased progressively over 8 hr, while 2.5 m*M* Ca suppressed PTH to low (Fig. 3) or undetectable levels (Fig. 2).

Validation of system. Previously we showed that CT release in this system was specifically stimulated by Ca, inhibited by metabolic poisons and unaffected by numerous other test agents (4). The present findings confirm this and provide similar evidence for the specificity and viability of the PTH secretory mechanism being studied. Figure 2 clearly shows that the system responds in the expected fashion with respect to release of both PTH and CT when changes in medium Ca are introduced. Table I further illustrates that secretion of both hormones *in vitro* requires an active secretory process, since release of both CT and PTH were inhibited when gland complexes were incubated in the presence of the metabolic poison, iodoacetate. Release of CT in 2.5 m*M* Ca was inhibited by ~75%, while PTH release in 1 m*M* Ca was inhibited by 90–100% (Table I).

TABLE I. INHIBITORY EFFECT OF IODOACETATE ON RELEASE OF IMMUNOREACTIVE CT AND PTH FROM RAT THYROPARATHYROID GLANDS INCUBATED FOR 8 hr.[a]

Group	CT, ng/ml	
	1 m*M* Ca	2.5 m*M* Ca
Control	0.83 ± 0.26	17.61 ± 3.40
Iodoacetate, 10^{-3} *M*	1.50 ± 0.62	4.43 ± 0.97*
	PTH, ng/ml	
	1 m*M* Ca	2.5 m*M* Ca
Control	6.95 ± 1.54	N.D.
Iodoacetate, 10^{-3} *M*	N.D.**	N.D.

[a] Each value represents mean ± SE for a group of 6 flasks. PTH and CT were measured in the same samples. N.D. = Not detectable (CT < 0.12 ng/ml, PTH < 0.49 ng/ml). *$P < 0.01$. **$P < 0.005$ vs Control at same [Ca].

Further evidence for the specificity of the two hormone secretory systems with regard

to control of secretion is provided by studies showing a number of unrelated test agents to be ineffective in altering secretion of CT and PTH. For example, Fig. 3 shows detailed results of an experiment where neurotensin and substance P both at 5 μg/ml failed to alter secretion of CT and PTH at either 1 m*M* Ca or 2.5 m*M* Ca, although the anticipated changes in CT and PTH in response to an alteration in medium Ca were clearly evident. The list of agents tested to date and found ineffective in altering concurrent secretion of CT and PTH from individual rat thyroparathyroid glands incubated *in vitro* for 8 hr includes, in addition to neurotensin and substance P, pentagastrin (5 μg/ml), secretin (6.7 U/ml), 1,25-$(OH)_2D_3$ (2.5 ng/ml) and 24,25-$(OH)_2D_3$ (2.5 ng/ml) and ethanol (5 μl/ml).

Discussion. Numerous studies of secretion of CT (4, 7–11) and PTH (12–17) *in vitro* have been reported. Generally these studies have involved incubation of only thyroid or ultimobranchial tissue to measure CT release (8–11) or incubation of only parathyroid tissue to measure PTH release (12–17). In the present study we have incubated entire rat thyroid and parathyroid glands together for up to 8 hr. Concurrent secretion of both CT and PTH from a single gland complex were evaluated using radioimmunoassays that measure rat CT and rat PTH. The results show that secretion of the two hormones varies inversely, CT being stimulated and PTH suppressed by 2.5 m*M* Ca and PTH being stimulated and CT suppressed by 1 m*M* Ca (Fig. 2). The secretory mechanisms are energy-requiring and not simple due to nonspecific leakage of hormone into medium, because the two hormones are affected in appropriate and opposing fashions by medium [Ca] and because both secretory mechanisms are inhibited by iodoacetate (Table I). Further evidence for specificity and reliability of the system resides in the fact that a number of different test agents failed to alter release of either CT or PTH at either 1 or 2.5 m*M* Ca. While higher doses of these agents might yield some effect, the ineffective doses tested already are superphysiological. However, further studies will be required before it can be concluded that these compounds are completely ineffective. These agents all are of some interest because: (a) secretin has been reported to affect bovine PTH release *in vitro* (16); (b) pentagastrin affects CT release in some species but not in the rat (4); (c) neurotensin and substance P are peptides with a variety of potent pharmacologic effects; and (d) possible effects of vitamin D metabolites on PTH release remain controversial and unresolved (18–22).

Although further studies will be required to examine secretion of rat CT and PTH in more detail, the present results show that simultaneous incubation of rat thyroid and parathyroid glands provides a novel *in vitro* system for studying concurrent secretion of PTH and CT and the mechanisms involved.

Summary. Thyroparathyroid glands were removed from 8-day-old baby rats and incubated *in vitro* for up to 8 hr. Release of both PTH and CT into medium were measured using immunoassays that measure rat CT and rat PTH. Results showed that release of CT and PTH *in vitro* were inversely related. At 1 m*M* Ca, PTH in medium increased progressively up to ~5 ng/ml while CT was only 1–2 ng/ml. At 2.5 m*M* Ca, PTH was suppressed to low levels (<0.5 ng/ml) while CT increased five- to tenfold (up to 10–20 ng/ml). Secretion of both CT and PTH were inhibited by 10^{-3} *M* iodoacetate; at 2.5 m*M* Ca, CT secretion was inhibited 75% and, at 1 m*M* Ca, PTH release was inhibited >90%. PTH and CT release were not altered by several other agents tested, including secretin, pentagastrin, 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$.

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