Proceedings of the National Academy of Sciences
Vol. 65, No. 2, pp. 446–453, February 1970

Effect of Thyrocalcitonin on Adenosine 3′:5′-Cyclic Phosphate Formation by Rat Kidney and Bone

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Communicated by Robert W. Berliner, October 28, 1969

Abstract. Thyrocalcitonin (TCT) increased the rate of accumulation of adenosine 3′:5′-cyclic phosphate (cyclic AMP) when added to incubations containing washed particles from whole rat kidney, adenosine triphosphate (ATP), $MgSO_4$, and caffeine. The maximum stimulatory effect of TCT, 44 ± 6.7 per cent, was always less than the 150 to 250 per cent increase produced by parathyroid hormone (PTH). The effect of both hormones together was no greater than that of PTH alone when each was present at a maximally effective concentration. Since neither TCT nor PTH altered the rate of degradation of cyclic AMP by the kidney preparation, it may be inferred that their effects on cyclic AMP accumulation are the result of increased formation of cyclic AMP. Adenyl cyclase activity in homogenates of renal cortex was stimulated to a greater extent by TCT and PTH than was that of medulla, whereas, as reported earlier, the effect of vasopressin was much larger with homogenates of medulla. The accumulation of cyclic AMP in incubations of rat kidney cortex slices was increased 20 to 60 per cent by TCT and 50 to 140 per cent by PTH. The accumulation of cyclic AMP in incubations of rat calvaria was increased about threefold with TCT and nine to tenfold with PTH, while reduced and alkylated TCT had less than 10 per cent of the activity of TCT. These observations are consistent with the view that the physiological effects of TCT and PTH in kidney and bone are secondary to the enhanced formation of cyclic AMP.

Introduction. A variety of hormones produce their characteristic effects by enhancing the formation of adenosine 3′:5′-cyclic phosphate (cyclic AMP)* in specific target tissues.[1–3] There is good evidence that the effects of parathyroid hormone (PTH) in bone and in kidney are mediated by cyclic AMP.[4–8] Thyrocalcitonin (TCT) is believed to have physiological effects on kidney as well as on bone.[9–12] Although Chase *et al.*[7] were unable to demonstrate any effects of TCT on adenyl cyclase in bone, as reported below, TCT and PTH increased the accumulation of cyclic AMP in incubations of calvaria from newborn rats. In addition, TCT enhanced the accumulation of cyclic AMP in slices and in cell-free preparations of rat kidney. A preliminary report of some of these studies has appeared in abstract form.[13]

Methods and Materials. Adenyl cyclase activity in cell-free systems: Kidneys were obtained from Osborne-Mendel rats (150–200 gm) and placed in chilled 0.25 *M*

sucrose. The capsules were removed, and the whole organ was homogenized in 4 volumes of 0.25 *M* sucrose in a glass tissue grinder. After centrifugation of the homogenate at 4000 × *g* for 20 min at 4°, the precipitate was suspended in 0.25 *M* sucrose and again collected by centrifugation as described above. The washed particles were dispersed by homogenizing in a volume of 0.25 *M* sucrose equal to that of the original homogenate. The protein content of the suspension was 10–15 mg/ml.[14] Samples of the particulate preparation were used immediately or rapidly frozen in a dry ice-ethanol bath and stored at −80° until needed. Storage for as long as 3 months resulted in no loss of adenyl cyclase activity (basal and hormone- or fluoride-stimulated). For some experiments the cortex and medulla were homogenized separately in 4 vol. of 0.25 *M* sucrose. These homogenates were used immediately or frozen and stored as described above.

The washed particulate preparations or homogenates were used as a source of adenyl cyclase to catalyze the formation of cyclic AMP from ATP. The reaction mixture contained in addition to the enzyme preparation, 40 mM Tris buffer, pH 7.8, 13 mM caffeine, 2 mM ATP, 6.6 mM $MgSO_4$, 10–15 μg bovine serum albumin, and other additions as indicated in a total volume of 0.6 ml. Samples were incubated at 30° with gentle shaking, usually for 10 min. Incubations were terminated by heating at 100° for 3 min. After centrifugation, the supernatant fractions were assayed for cyclic AMP using a liver phosphorylase activation assay system.[15]

Cyclic AMP accumulation in incubations of kidney cortex slices: Kidneys were kept in Krebs-Ringer bicarbonate medium (4°) containing glucose, 2 mg/ml, while slices of cortex were prepared. Slices were pooled and incubated in fresh medium of the same composition for 30 min at 37°. Four or five slices, ca. 200 mg, were blotted, weighed, and transferred to flasks containing 3 ml of fresh medium with or without theophylline and/or hormone(s). All incubations were performed in an atmosphere of 95% O_2, 5% CO_2. After incubation for 10–20 min, the contents of each flask (slices plus medium) were homogenized in 10 ml of 0.1 *N* HCl containing about 2500 cpm of [^{3}H] cyclic AMP (ca. 6 pmoles). Samples were heated at 100° for 10 min and neutralized. Two milliliters each of 0.3 *M* $ZnSO_4$ and 0.3 *M* $Ba(OH)_2$ were added. Samples were mixed and centrifuged. The $ZnSO_4$ and $Ba(OH)_2$ additions were repeated without disturbing the first precipitate. After centrifugation, the supernatant fractions were applied to Dowex-2 chloride columns for purification of cyclic AMP, and samples were assayed for tritium and cyclic AMP content as previously described.[15] The recoveries of [^{3}H] cyclic AMP (25–35%) were used to calculate the cyclic AMP content in incubations.

Cyclic AMP accumulation in incubations of calvaria: One- to three-day-old rats were anesthetized with ether. Calvaria were removed, divided into quarters, and incubated 30 min at 37° in Krebs-Ringer bicarbonate medium containing glucose, 2 mg/ml. Four or five pieces of calvaria, ca. 150 mg, were blotted, weighed, and transferred to 3 ml of fresh medium of the same composition with or without theophylline and/or hormones, and incubated 10 min. All incubations were performed in an atmosphere of 95% O_2, 5% CO_2. The cyclic AMP in each incubation (calvaria plus medium) was extracted and purified using methods similar to those described above, except that the $Ba(OH)_2$-$ZnSO_4$ precipitation was omitted. The cyclic AMP in Dowex-2 eluates was further purified on Dowex-50 columns as previously described.[16]

Materials: Bovine PTH (3000 units/mg) was obtained from Wilson Laboratories. The homogeneous preparation of bovine TCT[17, 18] contained 200 MRC units/mg as determined by the rat hypocalcemic assay of Cooper *et al.*[19] Reduced and alkylated TCT was prepared from pure bovine TCT as previously described.[20] Concentrated solutions of TCT, PTH, and the TCT derivative were prepared in 0.04 *N* acetic acid containing 200 μg/ml bovine serum albumin and stored at −20° until used. In all experiments the diluent used for the hormones was also added to control incubations and was without effect. Synthetic lysine vasopressin (ADH) was purchased from Calbiochem. Prostaglandin E_1 was obtained from Upjohn Co. through the courtesy of Dr. J. E. Pike. Other hormones and materials were obtained as previously described.[15, 21]

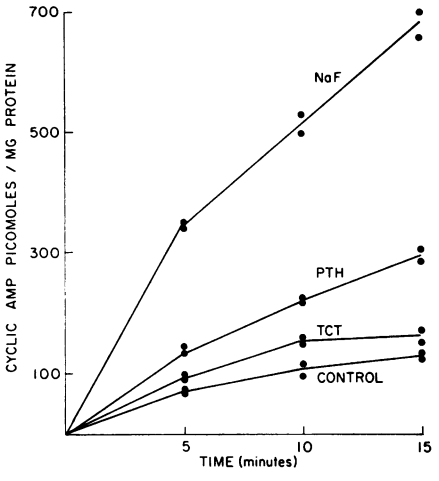

FIG. 1.—Effect of TCT, PTH, and NaF on cyclic AMP accumulation. Washed particles from whole kidney (0.8 mg protein) were incubated as described in **Methods and Materials.** The concentration of TCT and PTH was 10 μg/ml and of NaF 10 mM. Each point represents the value from one incubation.

Results. As shown in Figure 1, TCT, PTH, and NaF each increased the rate of accumulation of cyclic AMP in incubations containing washed particles from rat kidney. Under no conditions were the rates constant during the first 10 minutes, the incubation period used in most other experiments. Half-maximal effects of either hormone were produced with a concentration of 0.4–0.5 μg/ml (Fig. 2). In the experiment presented in Figure 2, the stimulation with 10 μg/ml of either hormone (not shown) was no greater than that with 5 μg/ml. In seven experiments (14 incubations) the effect of TCT at concentrations of 5–20 μg/ml was +44 ± 6.7 per cent (mean ±SE), whereas that of PTH ranged from 150 to 250 per cent. The pH optimum for both hormones was near 7.8 (Fig. 3).

The effects of TCT and PTH were additive when both hormones were present together at concentrations which individually produced less than maximal stimulation (Expts. 1 and 2, Table 1). However, when present together at concentrations which alone produced maximal effects, the amount of cyclic AMP accumulated was no greater than that seen with PTH alone, which represented about 40 per cent of the activity in the presence of 10 mM NaF (Expts. 3 and 4, Table 1). In the presence of NaF at this concentration, the activity of adenyl cyclase from a variety of tissue preparations is apparently maximal.[22]

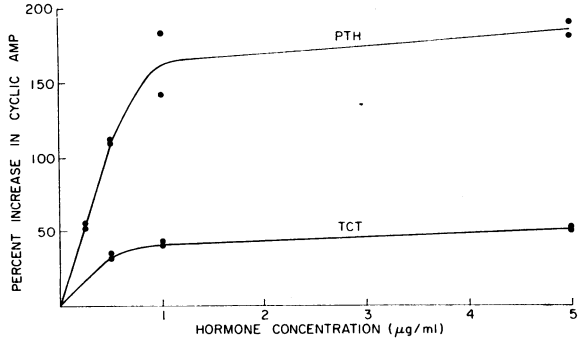

FIG. 2.—Relationship of cyclic AMP accumulation to TCT and PTH concentration. Washed particles from whole kidney (0.9 mg protein) were incubated 10 min. The cyclic AMP accumulated in control incubations was 0.12 mμmole/mg protein. Each point represents the value from one incubation.

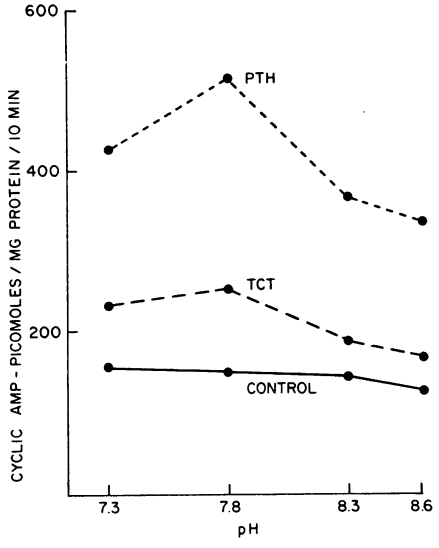

FIG. 3.—Effect of pH on response to TCT and PTH. Washed particles from whole kidney (1.4 mg protein) were incubated for 10 min in the presence of 40 mM Tris buffer at the pH indicated. The concentrations of TCT and PTH were 10 and 11 μg/ml, respectively. The means of values from duplicate incubations are reported.

TABLE 1. *Effects of TCT, PTH, and NaF on cyclic AMP accumulation.*

Expt.	Addition	Concentration (μg/ml)	Cyclic AMP accumulated (mμmoles/mg protein/10 min)
1	Diluent		0.14 (0.13, 0.14)
	TCT	5	0.22 (0.22, 0.22)
	PTH	3	0.35 (0.34, 0.35)
	TCT + PTH	5 + 3	0.41 (0.41, 0.41)
2	Diluent		0.12 (0.11, 0.12)
	TCT	5	0.17 (0.16, 0.17)
	PTH	1.8	0.27 (0.26, 0.28)
	PTH	6	0.35 (0.33, 0.36)
	TCT + PTH	5 + 1.8	0.35 (0.33, 0.36)
	TCT + PTH	5 + 6	0.39 (0.39, 0.39)
3	Diluent		0.14 (0.13, 0.14)
	TCT	10	0.22 (0.21, 0.22)
	PTH	9	0.41 (0.40, 0.41)
	TCT + PTH	10 + 9	0.42 (0.41, 0.43)
4	Diluent		0.12 (0.11, 0.13)
	TCT	20	0.19 (0.18, 0.19)
	PTH	20	0.29 (0.27, 0.30)
	TCT + PTH	20 + 20	0.30 (0.28, 0.31)
	NaF, 10 mM		0.75 (0.74, 0.75)

Washed particulate preparations from whole kidney (0.9–1.4 mg protein) were incubated for 10 min as described in **Methods and Materials**. Values reported are the means of duplicates which are presented in parentheses.

ADH (10 μg/ml) also increased the accumulation of cyclic AMP by particulate preparations from whole kidney (Table 2). Glucagon (8 μg/ml) and prostaglandin E_1 (5 μg/ml) had small stimulatory effects (25–35%), while L-epinephrine (0.1 mM) and ACTH (10 units/ml) were without effect. Other concentrations of these hormones were not tested. The significance of the small effects of glucagon and prostaglandin E_1 observed in two experiments was not further investigated.

TABLE 2. *Effects of other hormones on cyclic AMP accumulation.*

Addition (conc.)	Cyclic AMP accumulated (mμmoles/mg protein/10 min)
Diluent	0.13
PTH, 5 μg/ml	0.28
ADH, 10 μg/ml	0.20
Glucagon, 8 μg/ml	0.18
Prostaglandin E_1, 5 μg/ml	0.17
L-Epinephrine, 0.1 mM	0.14
ACTH, 10 units/ml	0.12

Washed particulate preparations from whole kidney (1-mg protein) were incubated 10 min as described in **Methods and Materials**, with and without various hormones at concentrations as indicated. The means of duplicate incubations are reported.

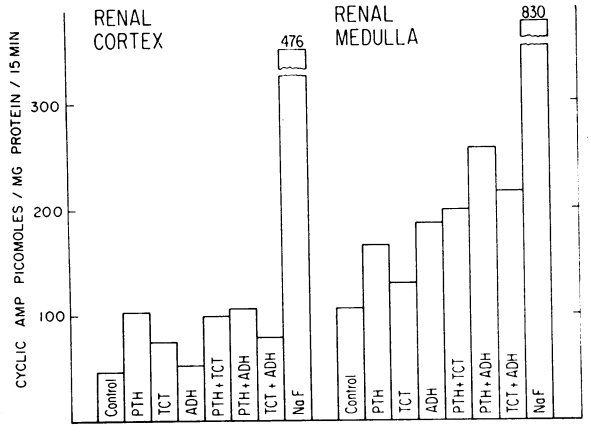

FIG. 4.—Effect of hormones and NaF on cyclic AMP accumulation in homogenates of cortex and medulla. Homogenate of cortex (3.5 mg protein) or medulla (2.2 mg protein) was incubated for 15 min. The concentrations of TCT, PTH, and ADH were 7.5, 11, and 10 μg/ml, respectively, and that of NaF 10 mM. The means of values from duplicate incubation mixtures are reported.

The results of the experiments summarized in Figure 4 are qualitatively similar to those of Chase and Aurbach[5] and in addition indicate that the effects of TCT, like those of PTH, are more marked on the enzyme activity in preparations from cortex than in those from medulla. ADH, 10 μg/ml, had no effect on the preparation from cortex when added alone or in the presence of TCT or PTH. However, the stimulatory effect of ADH on the enzyme from medulla was evident whether or not TCT or PTH was present. The adenyl cyclase activity of cortex and medulla preparations under all conditions examined was never greater than 20–30 per cent of that observed in the presence of 10 mM NaF.

The effects of these hormones on accumulation of cyclic AMP could result either from increased formation or decreased degradation of cyclic AMP. When

TABLE 3. *Effect of TCT and PTH on cyclic AMP degradation.*

Addition (conc.)	Residual cyclic AMP (mμmoles)
Diluent (no enzyme)	0.37
Diluent	0.36
PTH, 10 μg/ml	0.36
TCT, 10 μg/ml	0.36

Washed particles from whole kidney (1-mg protein) were incubated as described in **Methods and Materials**, except that ATP was omitted from the incubations and commercial cyclic AMP was added. After 10 min of incubation, samples were heated (100°) for 3 min and the supernatant fractions were assayed for residual cyclic AMP. Values reported are the means of duplicate incubations.

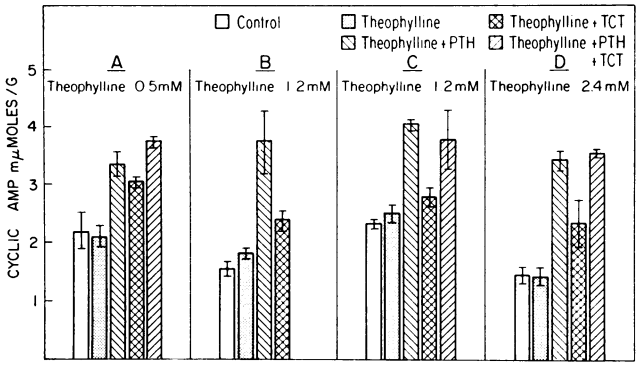

FIG. 5.—Effect of TCT, PTH, and theophylline on cyclic AMP content of cortical slice incubations. Slices were incubated for 20 min in Expt. A, 10 min in all others. The concentrations of TCT and PTH when present were 10 μg/ml except in Expt. A where they were 9 μg/ml. The height of the bar represents the mean and the vertical line the range of duplicate incubations.

washed particles from whole kidney were incubated under the standard conditions, but with commercial cyclic AMP added and ATP omitted to eliminate cyclic AMP formation, less than 5 per cent of the added cyclic AMP was degraded whether or not TCT or PTH was present (Table 3). Although these observations do not rule out the possibility that the hormones might stimulate an ATP-dependent pathway of cyclic AMP degradation, they are consistent with the conclusion that the effects of TCT and PTH on accumulation of cyclic AMP are the result of increased adenyl cyclase activity. TCT and PTH at the concentrations used in these studies had no effect in the assay for cyclic AMP. In several representative experiments, cyclic AMP from incubations with and without TCT or PTH was purified by Dowex-2 and paper chromatography (0.5 *M* ammonium acetate: ethanol, 2:5, v/v) before it was assayed in order to remove many nonspecific activators and inhibitors of the cyclic AMP assay system.[15] In this way it was established that the effects of TCT and PTH were in fact an accumulation of cyclic AMP and were not caused by variation in the amounts of other materials in the extracts assayed.

Effect of hormones on cyclic AMP accumulation in incubation of slices: Figure 5 summarizes the results of four experiments with slices of kidney cortex. TCT (10 μg/ml) produced a small but consistent increase in cyclic AMP accumulation which was less than that observed with PTH (10 μg/ml) and did not add to it. Higher concentrations of either hormone (20 μg/ml) were no more effective than 10 μg/ml. Theophylline at the concentrations tested, 0.5–2.4 mM, had little or no effect. In the absence of theophylline, no effect of TCT was observed, and that of PTH was markedly diminished (data not shown). The ability of theophylline to potentiate hormone effects on cyclic AMP accumulation has also been observed in studies of other investigators with other hormones and intact tissue preparations.[23, 24]

Effects of TCT, PTH, and theophylline on accumulation of cyclic AMP in

TABLE 4. *Effect of hormones and theophylline on accumulation of cyclic AMP in incubations of calvaria.*

Expt	Addition	Concentration (μg/ml)	Cyclic AMP Content mμmoles/gm wet wt	Cyclic AMP Content $\mu\mu$moles/mg protein
1	Diluent		0.4	
	TCT	10	1.2	
	PTH	10	3.5	
	TCT + PTH	10 + 10	3.9	
	Theophylline		0.8	
	Theophylline + TCT	10	1.5	
	Theophylline + PTH	10	5.3	
	Theophylline + TCT + PTH	10 + 10	7.4	
2	Diluent		0.5	10
	TCT	0.5	0.8	14
	TCT	5.0	1.4	26
	TCT	10.0	1.8	33
	PTH	0.5	2.8	53
	PTH	5.0	6.0	95
	PTH	10.0	4.9	100
	TCT + PTH	10 + 10	8.2	121
	TCT derivative	5.0	0.8	13

Pieces of calvaria were incubated as described in **Methods and Materials.** Theophylline when present was 1 mM. The values reported are the means of duplicate incubations.

incubations of calvaria: As shown in Table 4, both TCT and PTH increased the accumulation of cyclic AMP in incubations of calvaria from newborn rats whether or not theophylline was present. The percentage effects of the hormones were considerably greater in bone than they were in kidney, although the concentrations required were similar in the two tissues. In Expt. 2, after ten minutes with PTH (10 μg/ml), cyclic AMP content of the system was ten times that of the controls. The smaller effect of TCT (10 μg/ml) was demonstrable also in the presence of this apparently maximally effective concentration of PTH. Reduced and alkylated TCT had less than 10 per cent of the activity of TCT. In other studies this TCT derivative has been found to be biologically inactive in the rat hypocalcemic assay.[25]

Discussion. Although the apparently opposing effects of PTH and TCT on the metabolism of calcium and phosphate in bone have been extensively investigated, it is not clear that the two hormones act in an antagonistic fashion in the same cell type. It appears that the effects of TCT on bone, like those of PTH,[7, 8] are mediated by cyclic AMP. The findings reported above are consistent with the view that the two hormones act on different adenyl cyclases, probably in different types of bone cells.

In the kidney, PTH increases excretion of phosphate by stimulating adenyl cyclase activity in specific tubule cells. It is probable that TCT also has effects on renal metabolism, but these are less well defined. In studies of epithelial cells cultured from monkey kidney, Borle[11] found that PTH increased calcium influx (apparently passive) whereas TCT inhibited calcium efflux. Pak *et al.*[12] concluded that TCT acts directly on the kidney to increase the excretion of calcium, but not that of phosphate. The preparation of TCT used in these experiments was, however, only partially purified.

The observation that both TCT and PTH have larger effects on adenyl cyclase in homogenates of renal cortex than on that from medulla, and that stimulation by the two hormones together is no greater than that produced by PTH alone could most simply be interpreted as indicating that both hormones act on the same cyclase from the same population of cells. One might expect, if this were correct, that each of the hormones would increase cyclase activity to the same maximum, whereas the levels attained with PTH were always greater than those with TCT. In preparations from fat cells, however, although there is good reason to believe that there is a single adenyl cyclase derived from a homogeneous population of cells, different hormones do produce different maximal levels of activity.[16, 26, 27] The available information concerning the physiological effects of TCT and PTH on the kidney, however, leads one to conclude that these hormones act on different cell types in the tubule. In any case, it seems likely that the effects of TCT, as well as PTH, on the kidney are secondary to its stimulation of cyclic AMP formation. Further studies of the influence of TCT on renal metabolism and correlation of these effects with changes in cyclic AMP production and excretion will be necessary to establish the validity of this hypothesis.

The authors wish to thank Mrs. Ferol Lieberman and Miss Rosemary Ronan for their excellent technical assistance.

* Abbreviations used: cyclic AMP, adenosine 3′:5′-monophosphate; TCT, thyrocalcitonin; PTH, parathyroid hormone; ADH, antidiuretic hormone, vasopressin.

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