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Research Article

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These data indicate that dibutyryl cyclic 3',5' AMP produces effects similar to parathyroid hormone in thyroparathyroidectomized rats, and support the notion that 3',5' AMP is an intermediate in the mechanism of PTH action. However, the changes in magnesium and potassium excretion are different after dibutyryl cyclic 3',5' AMP infusion from those seen after PTH infusion. Also, theophylline was found to potentiate the action of smaller doses of dibutyryl 3',5' AMP, but not that of PTH.

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Effect of Dibutyryl Cyclic Adenosine 3',5'-Monophosphate, Theophylline, and Other Nucleotides upon Calcium and Phosphate Metabolism

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ABSTRACT The effect of dibutyryl cyclic adenosine 3',5'-monophosphate upon calcium and phosphate metabolism in thyroparathyroidectomized rats was undertaken in an effort to clarify the possible role of adenosine 3',5'-monophosphate (3',5' AMP) in parathyroid hormone action. The infusion of dibutyryl cyclic 3',5' AMP at a rate of 3 mg/hr into thyroparathyroidectomized rats leads to changes in calcium, phosphate, and hydroxyproline excretion, and calcium and phosphate concentrations in plasma that are qualitatively similar to those induced by parathyroid hormone given at a rate of 5 μ g/hr. The effect of dibutyryl cyclic 3',5' AMP upon calcium and hydroxyproline mobilization from bone is blocked by thyrocalcitonin administration in the same way that thyrocalcitonin blocks PTH effects. Other closely related nucleotides do not act in the same way.

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INTRODUCTION

The cyclic nucleotide adenosine 3',5'-monophosphate (3',5' AMP) has been implicated in the action of numerous polypeptide hormones (1-4) and more recently in the action of estrogen (5). Until recently, the actions of parathyroid hormone and thyrocalcitonin were thought not to involve the adenyl cyclase system (6). However, Wells and Lloyd (7) then reported that large doses of theophylline induced an elevation of plasma calcium in parathyroidectomized rats. They interpreted this as evidence in favor of the view that parathyroid hormone exerts its effects through the intermediate 3',5' AMP¹ because theophylline is a known inhibitor of the specific phosphodiesterase that hydrolyzes 3',5' AMP to adenosine 5'-monophosphate (5' AMP) (1). Their conclusion would only be valid if this were the sole effect of large doses of theophylline on metabolism. Also, a curious aspect of their study was the finding that theophylline did not potentiate the calcium-mobilizing effect of small doses of parathyroid hormone, although according to present thinking, if parathyroid hormone does act by increasing 3',5' AMP levels, then theophylline should potentiate its effects.

Their study was soon followed by that of Chase and Aurbach (8) who reported that the adminis-

¹ Abbreviations: TCT, thyrocalcitonin; PTH, parathyroid hormone; TH, theophylline; DB CAMP, N⁶-2'-O-dibutyryl adenosine-3',5'-phosphate; CAMP (3',5' AMP), cyclic adenosine 3',5'-monophosphate.

tration of parathyroid hormone led to a prompt rise in the excretion of 3',5' AMP in the urine of parathyroidectomized rats, and that this rise preceded the changes in phosphate excretion. On the other hand, it was also clear from some of their data that the rise and fall in urinary 3',5' AMP was more transient than the rise and fall of urinary phosphate after parathyroid hormone administration.

Because of these studies, we undertook an investigation of the effects of dibutyryl cyclic AMP, theophylline, and related nucleotides upon renal function and bone resorption in thyroparathyroidectomized rats. Dibutyryl cyclic AMP was used because it is thought to enter cells more readily, and to be destroyed less readily than the natural analogue 3',5' AMP (9).

METHODS

The preparation of thyroparathyroidectomized animals and their perfusion has been described in detail in previous publications (10-12). It involved the long-term perfusion of the conscious restrained rat. The animal received a hypotonic electrolyte solution containing 20 mM NaCl, 2.5 mM HCl, 10 mM glucose, 5 mM CaCl₂, 5 mM MgCl₂, and inulin at a rate of 3.5 ml/hr. Previous studies (10-12) have shown that fluid and electrolyte balance was

maintained in such animals for periods of 3 or more days. It has also been shown that with a constant infusion of inulin, the rate of its urinary excretion was a suitable measure of the glomerular filtration rate (12). Analytic methods, statistical evaluation of the data, and other aspects of the study were similar to those previously employed (10-12). Purified bovine parathyroid hormone and partially purified porcine thyrocalcitonin were prepared as previously described (13, 14). The thyrocalcitonin used in these studies was the trichloroacetic acid powder, which was a relatively crude preparation. However, in other experiments, rats have been given highly purified thyrocalcitonin that produced nearly identical effects as those seen with the cruder preparation used in the present studies. Dibutyryl cyclic AMP and the other nucleotides were obtained from Schwarz Bioresearch, Inc., Mt. Vernon, N. Y., and theophylline from Nutritional Biochemicals Corporation, Cleveland, Ohio. The drugs and hormones were dissolved in the standard electrolyte solution, adjusted to neutral pH, and infused at constant rates after a suitable control period. Routinely, the animals were perfused for 16-20 hr after surgery with the standard electrolyte solutions before a control period of 8-10 hr was begun. Hormone or drug infusion was then initiated and maintained for 12-16 hr. A blood sample was taken at the end of the period of infusion for analysis of calcium and phosphate. The urine was collected in timed aliquots and analyzed for calcium, phosphate, magnesium, potassium, inulin, and hydroxyproline by our standard methods (10-12, 15-17). At least four

TABLE I
A Comparison of the Effects of PTH, DB CAMP, and DB CAMP Plus TCT upon the Rates of Excretion of Calcium, Phosphate, Potassium, Magnesium, Inulin, and Hydroxyproline in Thyroparathyroidectomized Rats

		Rate of urinary excretion						
	<i>Hr</i>	Ca ⁺⁺	HPO ₄ ⁻	Mg ⁺⁺	K ⁺	Inulin	HOP	
		<i>μmoles/min</i>				<i>μg/min</i>		
Control*	2-6	0.76 ± 0.007	0.28 ± 0.06	0.17 ± 0.07	0.52 ± 0.11	1.68 ± 0.11	0.37 ± 0.04	
	6-10	0.65 ± 0.008	0.23 ± 0.08	0.16 ± 0.05	0.49 ± 0.13	1.67 ± 0.08	0.38 ± 0.05	
PTH	10-14	0.029 ± 0.005	0.78 ± 0.11	0.12 ± 0.04	0.69 ± 0.09	1.57 ± 0.07	0.41 ± 0.05	
DB CAMP		0.024 ± 0.004	0.71 ± 0.10	0.08 ± 0.02	0.71 ± 0.11	1.59 ± 0.06	0.36 ± 0.03	
DB CAMP plus TCT		0.030 ± 0.004	0.58 ± 0.09	0.11 ± 0.03	0.52 ± 0.09	1.52 ± 0.07	0.26 ± 0.03	
PTH	14-18	0.099 ± 0.008	1.11 ± 0.18	0.16 ± 0.05	1.06 ± 0.11	1.53 ± 0.07	0.59 ± 0.06	
DE CAMP		0.079 ± 0.007	1.26 ± 0.16	0.02 ± 0.01	0.91 ± 0.18	1.52 ± 0.05	0.42 ± 0.04	
DB CAMP plus TCT		0.021 ± 0.005	0.93 ± 0.11	0.14 ± 0.04	1.06 ± 0.11	1.53 ± 0.05	0.22 ± 0.04	
PTH	18-22	0.271 ± 0.011	0.92 ± 0.15	0.21 ± 0.05	0.82 ± 0.12	1.57 ± 0.09	0.68 ± 0.04	
DB CAMP		0.150 ± 0.009	1.31 ± 0.16	0.03 ± 0.01	1.19 ± 0.09	1.46 ± 0.11	0.47 ± 0.06	
DB CAMP plus TCT		0.020 ± 0.002	0.90 ± 0.13	0.16 ± 0.05	1.19 ± 0.15	1.68 ± 0.08	0.22 ± 0.02	
PTH	22-26	0.330 ± 0.016	0.87 ± 0.13	0.22 ± 0.06	0.68 ± 0.08	1.59 ± 0.10	0.64 ± 0.05	
DB CAMP		0.156 ± 0.008	1.29 ± 0.13	0.06 ± 0.01	1.31 ± 0.14	1.67 ± 0.14	0.53 ± 0.04	
DB CAMP plus TCT		0.021 ± 0.002	0.87 ± 0.11	0.17 ± 0.06	1.11 ± 0.18	1.48 ± 0.08	0.24 ± 0.04	

* Mean rates of excretion observed in all three groups are recorded during control. A group of seven animals were given PTH, seven were given DB CAMP, and six were given DN CAMP plus TCT. The data are reported as mean \pm SEM.

and, in most cases, from five to seven animals were used for each experimental group.

RESULTS

Parathyroid hormone, dibutyl cyclic adenosine monophosphate, and thyrocalcitonin. As reported previously (12), and confirmed in this study (Figs. 1 and 2 and Table I), the infusion of parathyroid hormone at a rate of 5 $\mu\text{g/hr}$ to a thyroparathyroidectomized animal led to an immediate rise in the rate of urinary phosphate and potassium excretion, and a fall in urinary magnesium excretion. Shortly thereafter, the excretion of both urinary calcium and urinary hydroxyproline-containing peptides rose without significant change in glomerular filtration rate (as measured by inulin excretion). These results are shown in Figs. 1 and 2 (left) by the light lines. As can be seen, during the continued infusion of hormone the phosphaturia, calciuria, and hydroxyprolinuria were sus-

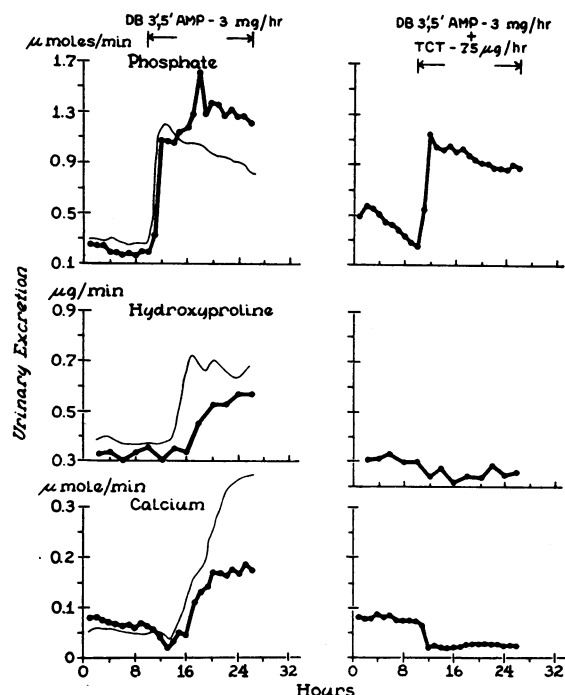


FIGURE 1 Left, the effect of PTH, 5 $\mu\text{g/hr}$, (—) and of dibutyl cyclic 3',5' AMP, 3 mg/hr, (●—●) upon the excretion of phosphate, hydroxyproline, and calcium in the urine of conscious, restrained thyroparathyroidectomized rats. Right, the influence of the simultaneous infusion of TCT, 75 $\mu\text{g/hr}$, upon the response of rats to DB CAMP, 3 mg/hr. The values represent the mean values obtained from five separate perfusions in each experimental group.

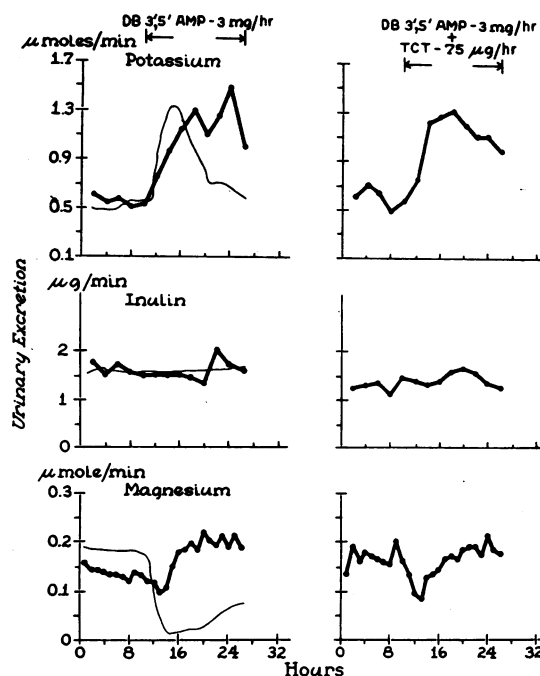


FIGURE 2 Left, the effect of PTH, 3 $\mu\text{g/hr}$, (—) and of dibutyl cyclic 3',5' AMP (●—●) upon the excretion of potassium, inulin, and magnesium in the urine of conscious, restrained thyroparathyroidectomized rats. Right, the influence of the simultaneous infusion of TCT, 75 $\mu\text{g/hr}$, upon the response of these rats to DB CAMP, 3 mg/hr. The results are from five animals in each group.

tained at high levels; magnesium retention continued, but the rate of K^+ excretion returned toward the control levels during the latter hours of hormone infusion.

The infusion of dibutyl cyclic AMP (DB CAMP) at a rate of 3 mg/hr led to changes that were qualitatively similar to those seen with PTH, except for the changes in Mg^{++} and K^+ excretion as shown by the heavy lines in Figs. 1 and 2 (left) and Table I. In the case of Mg^{++} , DB CAMP caused only a transient fall in Mg^{++} excretion, followed by a significant and sustained rise. Also, DB CAMP led to a less striking K^+ diuresis initially, but once achieved, the high rate of K^+ excretion was maintained throughout the perfusion, rather than returning to control levels as seen with PTH. A quantitative difference was also seen in the ratio of phosphate to calcium and hydroxyproline excretion. The infusion of DB CAMP at a rate of 3 mg/hr led initially to a very similar rate of phosphate excretion, like that seen with PTH

given at a rate of 5 $\mu\text{g/hr}$; however, DB CAMP resulted in a significantly lower ($P < 0.01$) rate of calcium and hydroxyproline excretion than that observed with PTH (Fig. 1 and Table I).

As reported previously (11) the infusion of a partially purified preparation of thyrocalcitonin, given simultaneously with PTH, to a thyroparathyroidectomized rat led to a striking decrease in both calcium and hydroxyproline excretion, and a significantly diminished phosphaturia when compared with that decrease seen when a similar dose of PTH was given alone. The simultaneous infusion of TCT with DB CAMP led to very similar effects upon the DB CAMP-induced responses (Figs. 1 and 2, right). TCT reversed the increase in both calcium and hydroxyproline excretion induced by DB CAMP and led to a definite decrease ($P < 0.02$) in phosphaturia when compared to that produced by DB CAMP alone. TCT infusion had no significant effect upon K^+ excretion, but it did block the increase in Mg^{++} excretion induced by DB CAMP (Fig. 2 and Table I).

The animals receiving either DB CAMP alone or DB CAMP with TCT showed no consistent change in inulin excretion (Fig. 2), although there was more variability than was seen in previous studies. However, the changes observed were not correlated with the changes in electrolyte excretion, nor were they of sufficient magnitude to account for these changes.

A surprising aspect of this study was the fact that DB CAMP led to no change in the rate of urine flow (Table II), even though cyclic 3',5' AMP is thought to be an intermediate in the anti-diuretic action of vasopressin upon the renal tubule (18).

When smaller doses of DB CAMP were infused, they produced no significant effect upon calcium

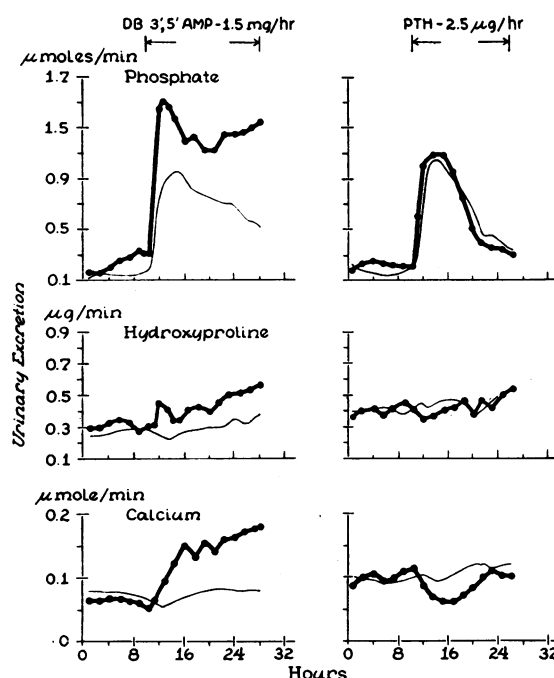


FIGURE 3 The effect of the infusion of PTH, 2.5 $\mu\text{g/hr}$, (right) or DB CAMP, 1.5 mg/hr, (left) upon the excretion of phosphate, hydroxyproline, and calcium in the absence (—) or presence (•—•) of theophylline given simultaneously at a rate of 0.50 mg/hr. The results are from four animals in each group.

and hydroxyproline excretion (Fig. 3), but continued to induce phosphaturia. A dose of 1.5 mg/hr of DB CAMP gave a renal response similar to 2.5 $\mu\text{g/hr}$ of PTH (Fig. 3), and this rate of PTH infusion produced no significant increase in either calcium or hydroxyproline excretion but still induced a significant ($P < 0.01$) phosphaturia and a significant ($P < 0.02$) initial fall in calcium excretion.

Theophylline, PTH, and DB CAMP. The effect of simultaneous theophylline administration upon the excretory patterns seen with submaximal doses of either PTH or DB CAMP was strikingly different in the two cases (Fig. 3). When animals were given 0.50 mg/hr of theophylline along with 2.5 $\mu\text{g/hr}$ of PTH, there was no significant difference in the rates of excretion of calcium, phosphate, or hydroxyproline from those rates seen in animals given PTH alone (Fig. 3). In contrast, the theophylline greatly potentiated the effect of a submaximal dose of DB CAMP (Fig. 3), which led to a very striking rise in both calcium and hydroxyproline excretion and to

TABLE II

Effects of Various Nucleotides upon Urine Volume and Inulin Excretion in the 1st hr after Infusion

	Urine flow	Inulin excretion
Control*	3.7 \pm 0.05 ml/min	1.61 \pm 0.07 $\mu\text{g/min}$
5' AMP	1.7 \pm 0.03 "	1.14 \pm 0.11 "
2',3' AMP	1.3 \pm 0.03 "	1.19 \pm 0.16 "
5' GMP	3.6 \pm 0.03 "	1.62 \pm 0.04 "
3',5' DB CAMP	3.6 \pm 0.04 "	1.59 \pm 0.06 $\mu\text{g/ml}$

* Each group consisted of four animals.

a greater and more sustained phosphaturia ($P < 0.01$). A smaller dose of theophylline (0.25 mg/hr) led to a significant increase in calcium and phosphate excretion but not hydroxyproline when given with DB CAMP, yet it had no effect when either given with PTH or alone.

Nucleotide specificity. The infusion of cyclic 3',5' AMP at a rate of 5 mg/hr gave a response similar to that seen with an infusion of DB CAMP at a rate of 1.5–2 mg/hr. The infusion of 5' AMP, guanosine 5'-phosphate (5' GMP), and the cyclic nucleotides 2',3' AMP and 2',3' GMP at rates of 5 mg/hr led to one of two patterns (Fig. 4). In the case of GMP and 2',3' GMP there was no change in calcium or hydroxyproline excretion, but a slight, but significant, increase ($P < 0.02$) in phosphate excretion was present (Fig. 4). Only the results with 5' GMP are shown. In the case of the adenine nucleotides, a slight, but definite ($P < 0.02$) and sustained, fall in urinary calcium excretion occurred (Fig. 4), along with an initial sharp decline in phosphate excretion, which, after 4–5 hr, showed a progressive rise in phosphate excretion (Fig. 4). When the guanine nucleotides were infused, there was no change in inulin excretion or urine volume (Table II) during nucleotide infusion. However, the adenine nucleotides led to a transient fall in urine flow and

inulin excretion (Table II) that persisted for 2–3 hr, whereupon both then returned to control values.

Plasma data. The effects of the infusion of PTH, DB CAMP, CAMP, theophylline, and the various nucleotides upon plasma calcium and plasma phosphate are summarized in Fig. 5. In the absence of any agent, plasma calcium was 5.7 ± 0.5 mg/100 ml and phosphate, 11.8 ± 0.6 . The infusion of PTH at a rate of $2.5 \mu\text{g/hr}$ led to a rise in plasma calcium to 8.0 ± 0.4 and a fall of plasma phosphate to 10.5 ± 0.5 mg/100 ml. Both changes were significant ($P < 0.01$). The simultaneous infusion of theophylline at a rate of 0.50 mg/hr and PTH at a rate of $2.5 \mu\text{g/hr}$ led to a plasma calcium of 8.2 ± 0.6 and a phosphate of 11.3 ± 0.6 mg/100 ml. These changes were not significantly different than those seen with PTH alone. When PTH was given at a rate of $5 \mu\text{g/hr}$, plasma calcium rose to 12.4 ± 0.6 and plasma phosphate fell to 8.6 ± 0.3 mg/100 ml. Both of these changes were significantly greater ($P < 0.01$) than the response seen with a rate of PTH infusion of $2.5 \mu\text{g/hr}$. When TCT ($75 \mu\text{g/hr}$) was given with PTH ($5 \mu\text{g/hr}$), plasma calcium rose to only 8.4 ± 0.4 , but plasma phosphate fell to 7.2 ± 0.5 mg/100 ml. Thus TCT significantly reduced the hypercalcemia ($P < 0.01$) induced

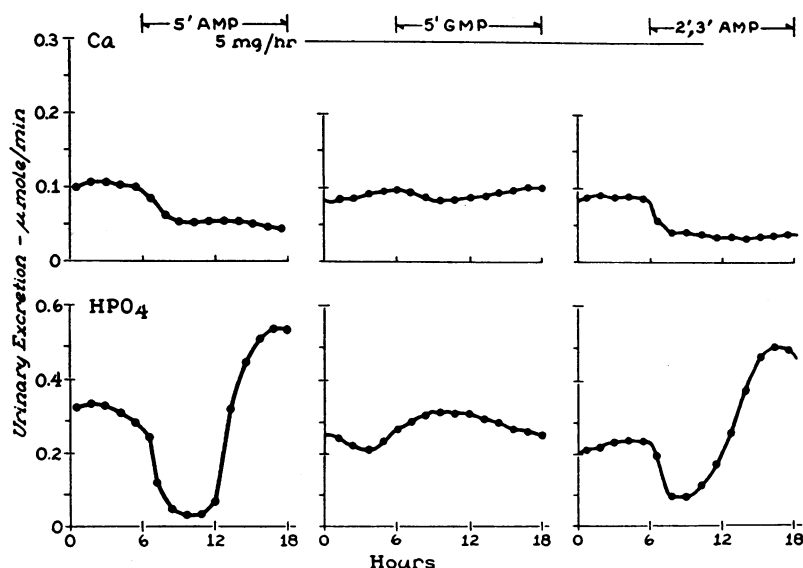


FIGURE 4 The effects of the infusion of 5' AMP, 5' GMP, and 2',3' AMP upon calcium and phosphate excretion in thyroparathyroidectomized rats. The results are from four animals in each group.

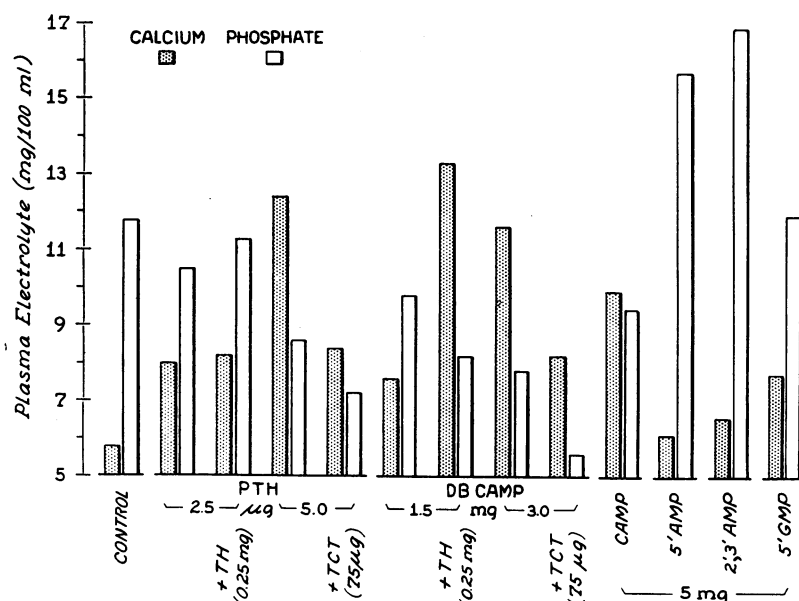


FIGURE 5 The plasma calcium and plasma phosphate values in control animals and those treated with one or more agents: PTH, parathyroid hormone; TH—thyrophylline; TCT, thyrocalcitonin; DB CAMP, dibutyryl cyclic 3',5' AMP; CAMP, cyclic 3',5' AMP. The results are from the same groups of animals reported in Figs. 1–4 and Table I.

by PTH, and enhanced the fall of plasma phosphate induced by PTH ($P < 0.02$).

The infusion of DB CAMP at a rate of 1.5 $\mu\text{g/hr}$ led to a rise in plasma calcium to 7.6 ± 0.4 , and a fall in plasma phosphate to 9.8 ± 0.4 mg/100 ml (Fig. 5). These are both significant changes from the control values ($P < 0.01$). The changes in plasma calcium and plasma phosphate induced by this dose of DB CAMP were not significantly different from those produced by PTH given at a rate of 2.5 $\mu\text{g/hr}$. When theophylline (0.25 or 0.5 mg/hr) was given with this dose of DB CAMP, plasma calcium rose to 13.3 ± 0.6 and plasma phosphate fell to 8.0 ± 0.5 mg/100 ml. These changes were significantly greater ($P < 0.01$) than those seen with DB CAMP alone. The infusion of DB CAMP at a rate of 3.0 mg/hr led to a rise in plasma calcium to 11.8 ± 0.7 and a fall in plasma phosphate to 7.8 ± 0.4 mg/100 ml. This change in plasma calcium is not different from that produced by PTH given at a rate of 5 $\mu\text{g/hr}$, but the fall in plasma phosphate with this dose of DB CAMP is probably significantly greater ($P < 0.10$). When TCT (75 $\mu\text{g/hr}$) was given with DB CAMP (3 mg/hr), the plasma calcium rose to only 8.2 ± 0.4 , but the plasma

phosphate fell to 5.6 ± 0.4 mg/100 ml. Thus TCT prevented the hypercalcemic effect of DB CAMP and enhanced the hypophosphatemia ($P < 0.01$). The infusion of 3',5' AMP at a rate of 5 mg/hr led to a rise in plasma calcium to 9.4 ± 0.6 and a fall in plasma phosphate to 9.1 ± 0.5 mg/100 ml. These changes were comparable to those induced by DB CAMP given at a rate of 2 mg/hr.

The infusion of either 5' GMP or 2',3' GMP at a rate of 5 mg/hr led to a slight rise in plasma calcium to 7.7 ± 0.5 without a significant change in plasma phosphate, 11.9 ± 0.7 mg/100 ml. (Fig. 5). The infusion of either 5' AMP or 2',3' AMP led to no significant increase in plasma calcium, 6.2 ± 0.5 , but a very significant rise ($P < 0.01$) in plasma phosphate, 16.9 ± 2.3 mg/ml.

DISCUSSION

The present data show that the infusion of dibutyryl 3',5' AMP into thyroparathyroidectomized rats leads to changes in excretion of calcium, phosphate, and hydroxyproline that are qualitatively similar to those observed after PTH administration (Figs. 1 and 2 and Table I). DB CAMP infusion also induces changes in plasma calcium and phosphate similar to those changes

seen with PTH (Fig. 5). A dose of 3.0 mg/min of DB CAMP produces an effect comparable to a dose of PTH at 5 μ g/hr (Figs. 1 and 5).

These effects of DB CAMP are relatively specific because other closely related nucleotides do not induce similar changes (Figs. 4 and 5). However, there are some qualitative and quantitative differences between the response to DB CAMP and PTH. For any given dose of DB CAMP a comparable dose of PTH, as judged by changes in plasma calcium and phosphate, leads to less phosphaturia and a greater mobilization of calcium and hydroxyproline from bone (Fig. 1 and Table I). There are also qualitative differences in the effects of the two agents upon K^+ and Mg^{++} excretion (Fig. 2). The change in the latter is particularly striking, with PTH causing a fall in Mg^{++} excretion and DB CAMP causing a significant rise. This difference in renal response to DB CAMP and PTH may be due to the fact that DB CAMP acts at several loci in the kidney, whereas PTH acts at a single locus.

Equally striking is the difference in the results when theophylline was given simultaneously with one or the other of the agents (Figs. 3 and 5). Theophylline greatly enhanced the response of the animal to DB CAMP, as judged both by the urinary excretion patterns (Fig. 3) and the changes in plasma calcium and phosphate (Fig. 5), but it had no effect upon the animal's response to PTH (Figs. 3 and 5). This difference in the effect of theophylline upon the response to DB CAMP and PTH might be explained if theophylline inhibited a plasma phosphodiesterase (leading to the destruction of DB CAMP), thereby allowing larger doses of DB CAMP to get to the target organs, particularly bone. The lack of effect of theophylline upon PTH action is less easy to explain unless one assumes that this dose of theophylline was insufficient to inhibit intracellular phosphodiesterase or, alternatively, that PTH does not activate an adenylyl cyclase but inhibits a phosphodiesterase. It may also be due to different changes in intra- and/or extracellular pH when PTH, rather than DB CAMP, is given, since Gulyassey and Edelman (19) have shown that the effects of theophylline are highly pH dependent.

In spite of the qualitative and quantitative differences in the responses to DB CAMP and PTH,

the similarities of their major effects tend to support the notions of Lloyd and Wells (7) and of Chase and Aurbach (8) that, 3',5' AMP is an intermediate in the action of parathyroid hormone (20). Our studies extend those of Chase and Aurbach, since their data concerned only the kidney. Our present data suggest 3',5' AMP may also be involved in the bone-mobilizing effects of PTH. This idea is further strengthened by the fact that TCT reverses the effects of DB CAMP on calcium and hydroxyproline excretion in the same way it reverses the effects of PTH (Fig. 1 and Table I). This observation immediately raises a question of theoretical interest. If PTH does act by enhancing 3',5' AMP production, and TCT blocks the effects of both PTH and DB CAMP, then TCT is not acting by blocking 3',5' AMP production, but must be acting either to enhance its destruction or be interacting with a later step in the sequence of events from 3',5' AMP production to bone mobilization.

Before finally concluding that 3',5' AMP is an intermediate, or the sole intermediate, in the action of PTH on bone and kidney, considerably more direct evidence must be obtained, and the qualitative differences in response to PTH and DB CAMP, observed in the present study, must be resolved.

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