PARATHYROID FUNCTION AND THE RENAL EXCRETION OF 3′5′-ADENYLIC ACID

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Cyclic 3′5′-adenylic acid (3′5′-AMP) has been found[1-4] in relatively high concentration in urine, but the physiological significance of this observation has not been completely elucidated. One report[3] indicated that the pituitary is not an important influence on the urinary excretion of cyclic adenylate in rats, and another[4] showed that in man excretion of the cyclic nucleotide is stimulated by injection of vasopressin and suppressed when secretion of this neurohypophyseal hormone is inhibited.

We have found that physiological amounts of parathyroid hormone cause a striking increase in urinary excretion of 3′5′-AMP through a direct action of the hormonal polypeptide on the kidney; this effect appears to be the earliest manifestation of the action of the hormone *in vivo*. These observations, outlined in this report, imply that the rate of secretion of parathyroid hormone is a major physiological factor affecting urinary 3′5′-AMP and suggest the possibility that cyclic adenylate is involved in the mechanism of action of the hormone.

Materials and Methods.—Cyclic 3′5′-AMP-H^3 (1000 mc/mmole) was obtained from Schwarz BioResearch; 3′5′-AMP-C^{14} (22 mc/mmole) was purchased from New England Nuclear Corp. Carrier-free purified radiophosphate was the product of Tracerlab Corp. Other reagents used, including the tricyclohexyl-ammonium salts of phosphoenolpyruvate and 3-phosphoglycerate, were the best grade available from standard suppliers. Stock crystalline preparations of pyruvate kinase, 310 U/mg; phosphoglycerate kinase, 250 U/mg; muscle glyceraldehyde-3-phosphate dehydrogenase, 40 U/mg; and myokinase, 360 U/mg, were dialyzed against 4000 ml of 10^{-3} *M* Tris-ethylenediaminetetraacetate (Tris-EDTA), pH 7.5, quick-frozen (acetone-solid CO_2 bath) in small portions, and stored at −20°. Cyclic nucleotide phosphodiesterase from bovine heart was purified according to Butcher and Sutherland[1] and stored in small aliquots frozen in liquid nitrogen; 1 μl of the enzyme solution catalyzed the conversion of 29.6 nmoles of 10^{-3} *M* cyclic adenylate to 5′-AMP in 30 min at 30° in 1 ml at pH 7.5. Pure bovine parathyroid hormone (PTH), 2000–3000 USP U/mg, was prepared as described previously.[5] Chromatographically purified corticotropin was the product of Sigma Chemical Co. Purified thyrocalcitonin was a gift of Dr. Paul Munson, and arginine vasopressin was the gift of Dr. Martin Petersen. Glucagon was purchased from Eli Lilly Co.

Male or female Sprague-Dawley rats weighing 140–200 gm were allowed free access to water and standard laboratory chow until the beginning of the experiment. The animals, conscious throughout the tests, were held in restraining cages and given intravenous fluid pumped at a constant rate, usually 5 ml per hour, through a catheter in the external jugular vein or a needle in a tail vein. Infusion at this rate was begun 12–16 hr before the experimental tests and could be continued for longer than 48 hr without causing edema, gain in weight, or changes in the electrolytes of the serum. The fluid given contained 4% glucose, 22 mM NaCl, and 5 mM $CaCl_2$; $CaCl_2$ was omitted in certain experiments in rats with intact parathyroid glands. Parathyroidectomy (PTX) was carried out by electrocautery before starting the continuous infusion. Urine was collected directly from male rats and through a No. 50 polyethylene catheter inserted into the bladder of female rats. Calcium was determined by atomic absorption spectrometry; the method of Taussky and Shorr[6] was used for phosphate.

Cyclic adenylate was detected enzymatically by converting it to 5′-AMP with phosphodiesterase and then to adenosine 5′-triphosphate (ATP) with myokinase-pyruvate kinase. The ATP thus

generated from 3′5′-AMP was measured by the P_i^{32}-ATP exchange reaction catalyzed by the coupled enzyme system triosephosphate dehydrogenase-phosphoglycerate kinase. This method is described in detail in another report.[7a] The procedure for urine is summarized here. To 1 ml of urine containing 0.025 μc of 3′5′-AMP-H^3 as a tracer were added 0.2 ml of 5% zinc sulfate and 0.2 ml of 0.3 *N* barium hydroxide; the final pH was 7.3 to 7.7.[7b] The supernatant solution was applied to a 0.5 × 3-cm column of Dowex-50, 100–200 mesh, in the hydrogen form; the column was eluted with water and the effluent collected in 1-ml fractions. Cyclic adenylate appeared in the third through seventh ml of eluate. Any one of the peak fractions, determined by testing for tritium, was assayed for cyclic adenylate. The lyophilized residue representing one fraction of the effluent was dissolved in 0.1 ml of 0.01% bovine serum albumin containing 1.8×10^{-3} *M* $MgCl_2$ and 0.04 *M* Tris-HCl at pH 8.0. Aliquots of this solution were incubated with 1 μl of purified nucleotide phosphodiesterase in 50 μl of 0.01% albumin, 1.8×10^{-3} *M* $MgCl_2$, and 0.04 *M* Tris-HCl at pH 8.0. After incubation at 37° for 20 min, the solutions were boiled for 3 min and cooled on ice. Then a test mixture for 5′-AMP was added in a total volume of 50 μl. This mixture contained: Tris-HCl pH 8.0, 0.16 *M*; 0.01% albumin; EDTA 2×10^{-4} *M*; pyruvate kinase, 80 μg/ml; phosphoglycerate kinase, 20 μg/ml; myokinase, 60 μg/ml; triosephosphate dehydrogenase, 200 μg/ml; phosphoenolpyruvate, 1.8×10^{-4} *M*; 3-phosphoglyceric acid, 4×10^{-4} *M*; cysteine as the free base, 4×10^{-3} *M*; magnesium chloride, 9.5×10^{-3} *M*; potassium chloride, 8×10^{-2} *M*; ATP, 2×10^{-10} *M*; and P_i^{32} (2×10^6 cpm/ml). After incubation for 30 min at room temperature, 1.3 ml of cold "precipitating solution"[8] was added to remove quantitatively inorganic radiophosphate from solution (the composition of the precipitating solution was 0.57 *N* perchloric acid–0.023 *M* ammonium molybdate–0.029 *M* triethylamine HCl, pH 5.0, containing 8×10^{-3} *M* ATP and 3.2×10^{-4} *M* inorganic phosphate). The samples were cooled on ice for 10 min, centrifuged in the cold, and 0.2 ml of the supernatant solution were taken to measure radioactivity. The supernatant solution contained ATP^{32} in proportion to the amount of cyclic AMP in the range of 0.006–0.25 nmole. Synthetic 3′5′-AMP was used as a standard in the assay. The fraction of 3′5′-AMP-H^3 found in the Dowex-50 effluent was used to calculate the total amount of cyclic adenylate in the original sample; 85–100% of the radioactivity was recovered in the effluent. Analyses were carried out in duplicate.

Results.—Small amounts of parathyroid hormone injected rapidly or as a sustained infusion caused a sharp rise in the rate of excretion of cyclic adenylate in the urine of parathyroidectomized rats (Figs. 1 and 2 and Table 1). It was frequently

TABLE 1

EFFECT OF PARATHYROID HORMONE ON 3′5′-AMP EXCRETION

Test rat	Infusion	Test condition	3′5′-AMP (nmoles/min) Control	Maximal response
a. PTX	C	PTH 2.5 μg	0.053 ± 0.007	0.118
		PTH 7.5 μg	" "	0.207
b. Normal	D/S + 20 mM $CaCl_2$	PTH 7.5 μg	0.092 ± 0.020	0.258
c. PTX	C	PTH 1.0 μg (PTH + H_2O_2 + catalase)	0.097 ± 0.020	0.092
		PTH 1.0 μg (H_2O_2 + catalase + PTH)	" "	0.174
d. PTX	C	PTH 0.5 μg	0.025 ± 0.006	0.047
	C + 2.2 mM theophylline	PTH 0.5 μg	0.078 ± 0.004	0.105
e. Normal	D/S	Before operation	0.215 ± 0.055	
		Sham operation	0.222 ± 0.046	
		PTX	0.107 ± 0.022	

Infusion solution D/S contained 4% dextrose and 22 mM NaCl; solution C contained D/S + 5 mM $CaCl_2$. Parathyroid hormone was injected intravenously in 0.15 ml over a 2-min period. Control represents the mean and standard deviation for at least three 30-min periods before giving parathyroid hormone. The maximal response occurred during the first 30-min collection period after PTH injection in each experiment. In expt. *c*, reagents were added in the order stated in parentheses. In expt. *e*, urine collections were begun 2 hr after the operations.

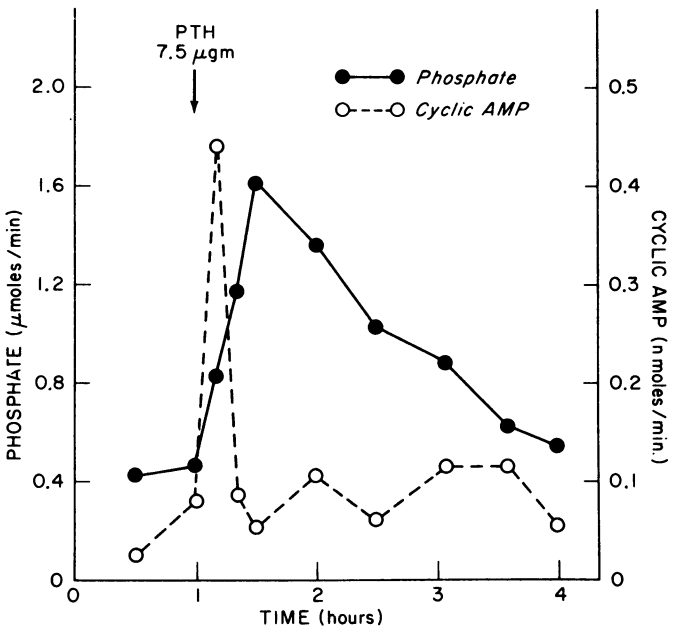

FIG. 1.—The effect of parathyroid hormone (*arrow*) on the excretion of phosphate and cyclic AMP (3′5′-AMP) in a PTX rat.

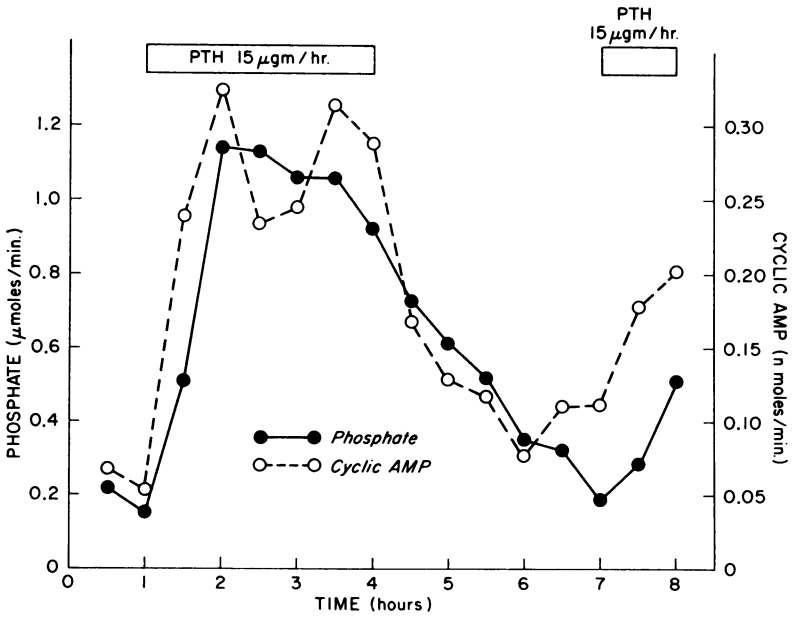

FIG. 2.—The effect of an infusion of parathyroid hormone on the excretion of phosphate and cyclic AMP (3′5′-AMP) in a PTX rat. PTH was added directly to the perfusing solution (see text).

observed that increased excretion of cyclic nucleotide preceded the well-known phosphaturic response to the hormone. After injection of the hormone, increased excretion of 3′5′-AMP was usually detectable in the first urine sample obtained, and was often maximal within 10 minutes. The highest rate was 5- to 20-fold that of the basal rate and diminished before phosphaturia became fully manifest. Parathyroidectomized rats given sustained infusions of hormone excreted 3′5′-AMP at a rate similar to that of normal rats; withdrawal of the hormone caused a fall in excretion to the rate observed in parathyroidectomized rats (Fig. 2). The response in urinary excretion of 3′5′-AMP appeared to be related to the dose of hormone (Table 1). In one experiment 200 μg of purified parathyroid hormone was infused over a 15-minute period into a human subject during the hypoparathyroid phase following removal of a parathyroid adenoma. A 33-fold rise (from 3.0 to 97.6 nmoles/min) in the excretory rate of 3′5′-AMP was measured during the first half hour after starting the infusion.

Uniformly labeled 3′5′-AMP-C^{14} was given intravenously to rats in order to determine whether the hormonal action might be effected merely by increasing the renal clearance of the cyclic nucleotide from the blood. The half life of the nucleotide in the circulation was approximately two minutes and only 20 per cent of that leaving the blood appeared in the urine. As shown in Figure 3 there was no increase in the clearance of 3′5′-AMP-C^{14} from the blood into the urine even though the hormone caused a tenfold increase in total excretion of cyclic nucleotide. Tests with the purified cyclic nucleotide phosphodiesterase showed that at least 80 per cent of the radioactivity found in the urine represented unchanged 3′5′-AMP.

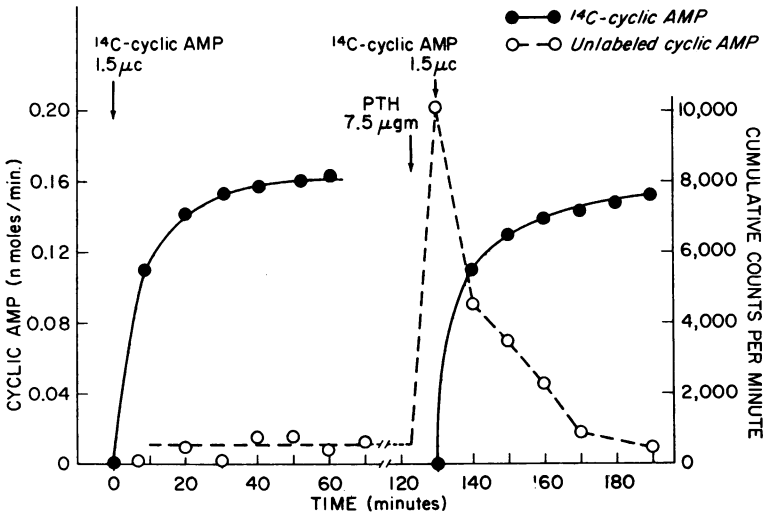

FIG. 3.—The effect of parathyroid hormone on the renal clearance of cyclic AMP (3′5′-AMP) in a PTX rat. Arrows represent the time of injection of C^{14}-cyclic AMP or parathyroid hormone. C^{14}-cyclic AMP measured in the urine is plotted as cumulative counts per minute. Unlabeled cyclic AMP represents the total urinary excretion measured by the assay described in the text minus that accounted for as C^{14}-cyclic AMP calculated from radioactivity excreted during each collection period and the known specific activity of C^{14}-cyclic AMP. Points plotted represent the end of each collection period.

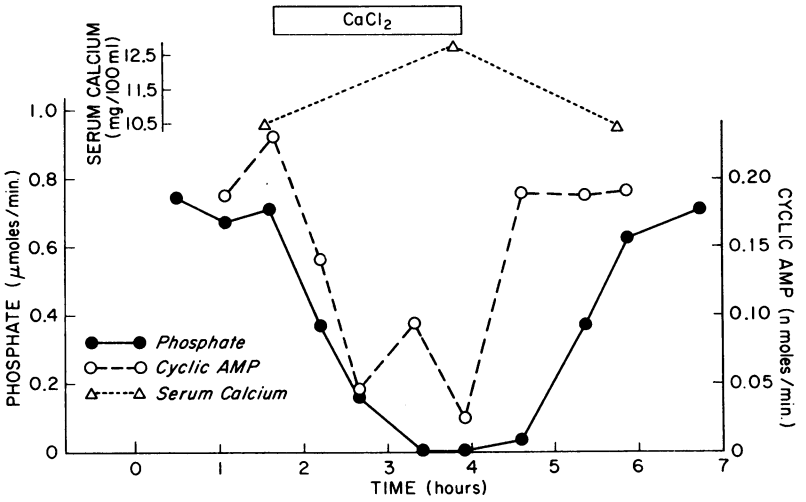

FIG. 4.—The effect of infusing calcium chloride on the excretion of phosphate and cyclic AMP (3′5′-AMP) in a normal rat.

Studies were carried out to determine the effect of interrupting the secretion of the hormone in normal rats. Parathyroidectomy caused a decrease in the rate of excretion of 3′5′-AMP (Table 1, expt. *e*). In another experiment 20 mM $CaCl_2$ was infused into a normal rat to study the effects of physiologically inhibiting endogenous secretion of the hormone. It is known that high concentrations of calcium in the blood inhibit and low concentrations stimulate the secretion of parathyroid hormone.[9, 10] An infusion to raise the serum calcium from 10.4 to 12.8 mg/100 ml caused a rapid decrease in excretion of cyclic adenylate as well as a reduction in phosphaturia (Fig. 4), the latter effect being well known. When the infusion of calcium was stopped and the blood calcium fell toward normal, there was a sharp rise in the excretion of cyclic adenylate which preceded the rise in phosphaturia. Both of the latter phenomena were undoubtedly effected by the return of secretion of parathyroid hormone as the blood calcium fell; the rise in excretion of cyclic adenylate appeared to be the first manifestation of this renewed secretion of the hormone. The hormone also increased 3′5′-AMP in the urine of a normal rat in which endogenous secretion of hormone was suppressed by a calcium infusion (Table 1, expt. *b*). Inactivated parathyroid hormone, prepared by oxidizing 1 μg of the polypeptide with 0.1 *M* hydrogen peroxide for one hour, caused no increase in the excretion of cyclic AMP. Catalase was used to destroy excess peroxide before injecting the preparation. A control reaction consisted of an identical solution of peroxide to which catalase was added before parathyroid hormone; this mixture caused a doubling of the rate of excretion of cyclic AMP (Table 1, expt. *c*).

Theophylline, an inhibitor of 3′5′-AMP phosphodiesterase[1] infused at a constant rate into parathyroidectomized rats, caused a sustained rise in excretion of 3′5′-AMP. Injection of parathyroid hormone during the infusion of theophylline caused a further increase, but the hormonal effect was not potentiated by the drug in the dose used (Table 1, expt. *d*).

TABLE 2

EFFECT OF POLYPEPTIDES ON 3′5′-AMP EXCRETION

Polypeptide	Dose*	3′5′-AMP (nmoles/min) Control	Response	Observed physiological effect
Thyrocalcitonin	12 μg/hr	0.147 ± 0.032	0.155	Serum calcium fell from 8.5 to 6.5 mg/100 ml
Vasopressin	0.1 U	0.094 ± 0.013	0.101	
	0.25 "	0.087 ± 0.028	0.082	Decrease in urine volume
	1.0 "	0.040 ± 0.011	0.067	Phosphaturia
	2.0 "	0.076 ± 0.003	0.247	
ACTH (pure)	600 mU	0.059 ± 0.013	0.070	Corticosteroid secretion
ACTH (impure)	600 mU	0.108 ± 0.013	0.265	—
Glucagon	0.4 μg	0.108 ± 0.015	0.129	Blood glucose rose from 106 to 157 mg/100 ml
C_2	4.0 μg	0.088 ± 0.023	0.105	—

Thyrocalcitonin was given by constant infusion for 2 hr. Other polypeptides were injected intravenously in 0.15 ml over a 2-min period. Control represents the mean and standard deviation for at least three collection periods before giving the polypeptide. Response represents the maximal excretory rate observed in any one period following polypeptide injection. Collection periods were 30 min.

* Each dose was tested in a separate rat.

Several other polypeptides (Table 2) were tested for possible influence on the rate of excretion of 3′5′-AMP. Vasopressin, 0.1 and 0.5 U, caused no increase in excretion of 3′5′-AMP. A slight increase was observed with 1.0 U and a marked increase with 2.0 U. Reduced urinary volume and phosphaturia were observed with each dose. The amounts of vasopressin given exceeded the physiological; 5–50 μU of vasopressin would be expected to cause a physiological effect on urine flow in rats of this size. Neither glucagon nor thyrocalcitonin caused a detectable change in the excretion of the cyclic nucleotide. A large dose, 600 mU, of a commercial oxycel preparation of corticotropin (ACTH) augmented the excretion of both phosphate and 3′5′-AMP. This response did not occur with chromatographically purified corticotropin in the same dose; it resembled that effected by vasopressin, and therefore it was attributed to contamination of the cruder commercial material with vasopressin. An ancillary observation was that Munson's purified thyrocalcitonin did not induce the phosphaturic response reported with other preparations even though the blood calcium fell 2 mg/100 ml. Perhaps the phosphaturia observed previously was a function of impurities in the preparations[11, 12] used. Protein C_2,[13] a biologically inert protein in crude parathyroid extracts, was completely inactive insofar as effects on cyclic AMP or phosphaturia were concerned.

Discussion.—The findings cited here lead to the conclusion that parathyroid hormone effects a major physiological influence on the urinary excretion of 3′5′-AMP, the rate of excretion of the nucleotide being related to the rate of secretion of the hormone. This influence of the hormone on urinary cyclic adenylate is brought about through a direct action on cellular metabolism of the kidney and not, as shown by experiments with 3′5′-AMP-C^{14}, through altered clearance of the nucleotide from the circulation. This effect must then be attributable to stimulation of the synthesis and/or release of intracellular 3′5′-AMP by cells of the kidney itself. The best hypothesis at present is that the hormone directly stimulates adenyl cyclase, the enzyme catalyzing conversion of ATP to cyclic AMP, as has been described for some other peptide hormones acting on other tissues.[14] In some of the latter instances it has been possible to test directly for hormonal stimulation of adenyl cyclase after fractionating cells and partially isolating the enzyme which appears to be an integral part of the cell membrane. This approach should be investigated for

parathyroid hormone and renal adenyl cyclase. It would be important also to determine whether 3′5′-AMP activates a particular enzyme within the kidney that would explain a series of biochemical reactions leading to the elimination of phosphate from the cell.

Several tests were made of the specificity of the response. Parathyroid hormone inactivated by oxidation was without effect. The biologically inert basic protein C_2 was also inactive in tests measuring phosphate and 3′5′-AMP. This was an important control substance since it has been found to mimic the action of parathyroid hormone in nonspecific[13] *in vitro* tests on ion transport and respiration of mitochondrial suspensions.

Vasopressin, the neurohypophyseal hormone regulating body fluids by a direct action on renal adenyl cyclase,[15] was the only polypeptide tested other than parathyroid hormone that influenced excretion of 3′5′-AMP in the rat. The amount of vasopressin required exceeded the physiological range and was over 50-fold greater relative to body weight than the amount eliciting increased urinary 3′5′-AMP in man.[4] The low sensitivity of the rat to the effects of vasopressin makes it unlikely that the latter hormone was a significant influence in our experiments with parathyroid hormone. Moreover, the secretion of vasopressin was probably suppressed by the infusions given. Studies on man[4] have shown that the excretion of 3′5′-AMP diminishes when sufficient fluids are given to inhibit the secretion of vasopressin.

Corticotropin and glucagon, polypeptide hormones that regulate specific physiological functions by activating adenyl cyclase in adrenal and liver, respectively,[14] are not considered physiological regulators of renal function and were not found to influence renal excretion of 3′5′-AMP. Even if cells sensitive to these polypeptides ordinarily release cyclic adenylate into the circulation, there may be no remarkable influence on urinary 3′5′-AMP since, as shown by experiments with 3′5′-AMP-C^{14}, only 20 per cent of the nucleotide in the blood reaches the urine. In rats deficient in both parathyroid hormone and vasopressin, the low rate of excretion of 3′5′-AMP may represent in part the fraction of the cyclic nucleotide that reaches the kidney after elaboration from tissues sensitive to other hormones. In the normal rat parathyroid hormone seems to be the most important physiological factor influencing the urinary excretion of 3′5′-AMP.

The finding that man responds similarly to parathyroid hormone with an increase in urinary cyclic AMP may lead to a useful test to differentiate the hypercalcemia of hyperparathyroidism from that of nonparathyroid disorders. An elevated excretion of cyclic AMP would be expected in hyperparathyroidism as contrasted to suppressed excretion in other hypercalcemic states. Clinical studies are in progress to test this hypothesis.

The most important physiological function of parathyroid hormone is the regulation of calcium in the extracellular fluids. This function is presumably mediated by influencing calcium ion transport through a direct action of the hormone on cell membranes. One might now consider whether a relationship exists between the hormone-sensitive membrane functions of cation transport and adenyl cyclase. One theory on the mechanism of action of parathyroid hormone has involved the concept that the hormone acts on the mitochondrial membrane[16] to mediate ion transport, and indeed respiration and ion transport activities of kidney and liver mitochondria are sensitive to parathyroid hormone added *in vitro*.[13, 16] However,

adenyl cyclase is usually considered an integral part of the plasma membrane rather than the mitochondrial membrane. Adenyl cyclase of liver[14] and kidney[17] has been found specifically in cell membrane fractions distinctly more dense than mitochondria. It is the plasma membrane fraction of kidney that has been found rich in vasopressin-sensitive adenyl cyclase.[17] If renal adenyl cyclase sensitive to parathyroid hormone is also located in the plasma membrane, it would become difficult to envisage the hormone as acting directly on adenyl cyclase at the plasma membrane as well as on cation transport at the mitochondrial membrane.

Activation of phosphate transport in the kidney has been considered as an explanation for the phosphaturic effect of parathyroid hormone.[16] The finding that cyclic adenylate is excreted before phosphate raises the possibility that phosphaturia represents a secondary effect mediated by 3′5′-AMP within the renal cell rather than an action directly on transport of phosphate. In any event, further attempts to resolve the mechanism of action of parathyroid hormone must take into account the temporal primacy of the cyclic nucleotide response described here.

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