EFFECTS OF PYROPHOSPHATE AND DIPHOSPHONATES ON PARATHYROID HORMONE- AND FLUORIDE-STIMULATED ADENYLATE CYCLASE ACTIVITY

R. PILCZYK, H. SUTCLIFFE and T.J. MARTIN*
University of Melbourne, Department of Medicine, Austin Hospital, Heidelberg, Victoria, 3084, Australia

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1. Introduction

Parathyroid hormone (PTH) promotes adenylate cyclase activity in renal cortex [1, 2] leading to an increased concentration of cyclic adenosine 3′5′-monophosphate (cAMP) in renal parenchymal cells [3, 4]. This cyclic nucleotide is proposed as an intermediate in the action of PTH [1, 2, 4, 5]. In the present experiments a study was made of the effects of diphosphonates and pyrophosphate on adenylate cyclase activity, since pyrophosphate is an end-product of the reaction. Diphosphonates are compounds related to pyrophosphate, but contain P–C–P bonds instead of the P–O–P bond of pyrophosphate [6]. They are extremely resistent to chemical and enzymatic hydrolysis [6, 7], but otherwise have many of the properties of pyrophosphate [6–9].

2. Methods

Adenylate cyclase preparations were made from kidney cortex essentially by the method of Marcus and Aurbach [10], modified by including 4.5 mM $MgCl_2$ and 30 mM KCl in the homogenizing medium (0.05 M Tris-HCl, pH 7.4, containing 10% dimethylsulfoxide). Adenylate cyclase activity was assayed by measuring the formation of ^{32}P-labelled cAMP from (α-^{32}P) ATP in incubations which contained the following in a total volume of 80 μl: 50 mM Tris-HCl, pH 7.4; 0.5 mM (α-^{32}P) ATP (specific activity approx. 20 cpm per pmole); 1 mM cAMP; 0.013% BSA; 30 mM KCl; 4.5 mM $MgCl_2$; phosphoenolpyruvate 2.5 mM; pyruvate kinase 1.5 I.U.; 300 μg protein of the kidney cortex preparation, and test substances. After 10 min incubation the reaction was stopped by adding excess ATP and boiling, and the method of Ramachandran [11] was used to purify the generated cAM^{32}P. Using ^{3}H-labelled cAMP, recoveries of 80–95% were obtained, and the coefficient of variation for the adenylate cyclase assay method determined for each new batch of enzyme, varied from 3 to 7%.

(α-^{32}P) ATP and (^{3}H) cAMP were purchased from the Radiochemical Centre, Amersham, U.K. Dichloromethylenediphosphonate (Cl_2DP) and ethane-1-hydroxy-1,1-diphosphonate (EHDP) were kindly provided by Dr. M.D. Francis, Procter and Gamble Co., USA. All other chemicals were from standard suppliers.

PTH was prepared in our laboratory by the method of Aurbach [12] to the stage of TCA precipitation. This preparation (TCA-PTH) had a potency of 320 units per mg [10].

3. Results

Dichloromethylenediphosphonate and pyrophosphate inhibited fluoride- and PTH-stimulated adenylate cyclase activity. Table 1 shows the results of an experiment in which pyrophosphate inhibition was virtually complete at 10 mM, and at 1 mM was only slightly greater than the inhibition produced by

* To whom to address correspondence.

Table 1
Effects of dichloromethylenediphosphonate (Cl_2DP) and pyrophosphate on NaF- and PTH-stimulated kidney adenylate cyclase activity (pmoles cAMP formed in 10 min).

Treatment	Control	Cl_2DP		Pyrophosphate	
		0.2 mM	0.02 mM	10 mM	1 mM
Nil	14	12	16	2	14
12 μg TCA-PTH	62	46	63	7	42
10 mM NaF	202	63	122	3	38
12 μg TCA-PTH + 10 mM NaF	281	109	163	2	48

Each result represents the mean of triplicate determinations, individual results varied by no more than 10% from the mean.

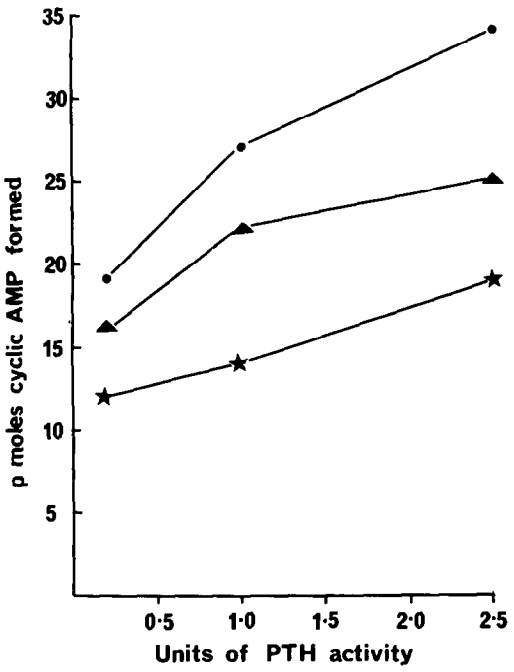

Fig. 1. Effects of 1 mM pyrophosphate (▲—▲—▲) and 0.35 mM dichloromethylenediphosphonate (★—★—★) on PTH-stimulated kidney adenylate cyclase activity compared with control (●—●—●). Results shown represent the mean of triplicate determinations; individual results varied by no more than 10% from the mean.

0.2 mM Cl_2 DP. Both the diphosphonate and pyrophosphate influenced the fluoride- to a greater extent than the hormone-stimulated activity.

PTH and fluoride effects were found to be additive. In incubations in which fluoride stimulation was inhibited by pyrophosphate or Cl_2DP, PTH was able to increase further the adenylate cyclase activity. In all experiments the concentration of diphosphonate required for inhibition was less than that of pyrophosphate. An example is given in fig. 1, showing the response to three dose levels of PTH in the presence of pyrophosphate and Cl_2DP.

The results in table 2 show that quantitative responses varied with different diphosphonates. Under most conditions studied, Cl_2DP was significantly more effective than EHDP in inhibiting fluoride-stimulated adenylate cyclase activity. Similar results were obtained with PTH-stimulated enzyme activity.

To determine whether cAMP could influence the enzyme activity, an experiment was carried out in which cAMP was omitted from the incubation medium, and theophylline (10 mM) used as a phosphodiesterase inhibitor. Addition of cAMP up to 4 mM had no effect on fluoride-stimulated adenylate cyclase activity, nor did it modify the inhibition produced by pyrophosphate (fig. 2).

4. Discussion

These experiments indicate that pyrophosphate, one of the products of the adenylate cyclase reaction, can influence the rate of cAMP accumulation, whereas the cyclic nucleotide itself appears to have no such effect. The fact that the pyrophosphate concentrations required to produce this effect are greater than the diphosphonate concentrations, may be explained by the presence of pyrophosphatase activity in the crude tissue preparations. Whereas pyrophosphate is susceptible to rapid enzymic hydrolysis, diphosphonates are extremely resistant. The actual concentrations

Table 2
Comparison of dichloromethylenediphosphonate (Cl_2DP) and ethane-1-hydroxy-1,1-diphosphonate (EHDP) effects on basal and fluoride-stimulated adenylate cyclase (pmoles cAMP formed; means ± SEM; 4 determinations for each point).

Treatment		(0.03 mM)			(0.3 mM)			(1.5 mM)		
		Cl_2DP	EHDP	p	Cl_2DP	EHDP	p	Cl_2DP	EHDP	p
Nil	24.9 ±0.6	16.1 ±0.9	20.4 ±1.3	NS	13.9 ±0.8	18.7 ±0.7	< 0.05	5.5 ±0.6	11.4 ±1.0	< 0.001
NaF (10 mM)	375.4 ±12.6	239.2 ±6.0	290.2 ±7.3	NS	180.5 ±2.3	228.3 ±16.3	< 0.05	78.9 ±4.1	139.8 ±4.4	< 0.001

Student's "t" test was carried out to test for significant differences between response to each dose level of diphosphonate. (NS: not significant).

of diphosphonates which were effective were small compared with the ATP (0.5 mM) in the incubations, and the inhibitory effects of pyrophosphate and diphosphonates cannot be explained by chelation of Mg ions, which are necessary for adenylate cyclase activity.

It has been proposed that pyrophosphate and the diphosphonates produce their effects by physicochemical means [9]. Diphosphonates have recently been found to reduce ^{45}Ca efflux from mitochondria previously labelled with the isotope [13]. Their influence on the adenylate cyclase reaction is a further metabolic effect which may be of some general importance, particularly in view of the proposed use of diphosphonates in certain diseases in man [14].

The recent demonstration [15] that pyrophosphate infusion in rats blocked the effects of PTH but not those of cAMP provide *in vivo* evidence that pyrophosphate may play a regulatory role at least in PTH-stimulated adenylate cyclase systems.

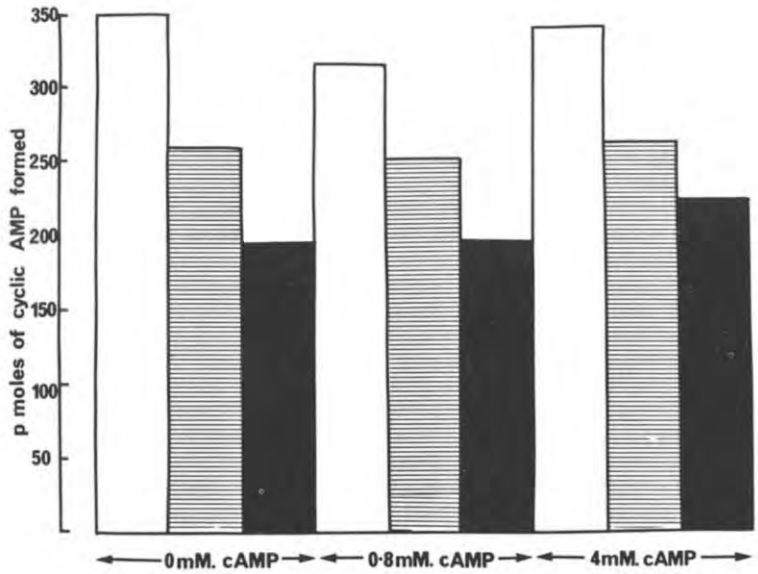

Fig. 2. Effect of cAMP on fluoride-stimulated adenylate cyclase activity in kidney. Each test mixture contained 10 mM NaF. (□): No added pyrophosphate; (▪): 1 mM pyrophosphate; (■): 2 mM pyrophosphate. Results shown represent the mean of triplicate determinations; individual results varied by no more than 10% from the mean.

Acknowledgement

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