CYCLIC-AMP ACTIVATES AND CALCIUM INHIBITS PROTEIN KINASE ACTIVITY IN AVIAN PARATHYROID GLANDS

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1. Introduction

It is generally accepted that calcium is the primary physiological modulator of parathyroid hormone (PTH) secretion [1]. In addition, several secretagogues such as dopamine [2], prostaglandins of the E series [3], β-agonist [4] and cholera toxin [5] also stimulate PTH secretion, probably through modulation of cyclic AMP levels within the parathyroid cell [2–4]. Little is known of the events following cyclic AMP production and intermediate to hormone release within the parathyroid cell.

It was suggested [6,7] that all the biochemical and physiological effects of cyclic nucleotides are mediated through specific protein kinases which promote the phosphorylation of specific proteins. This was confirmed in various tissues including endocrine glands [8,9].

Here, cyclic AMP-dependent protein kinase activity was demonstrated in avian parathyroid gland cells. The activity of this protein kinase was shown to be modified by calcium concentration.

2. Materials and methods

2.1. *Animals and parathyroid glands*

Vitamin D-deficient chicks were raised and maintained as in [10] for 4–5 weeks. The parathyroid glands were removed into ice-cold Eagle's medium containing 1 mM $CaCl_2$ and 20 mM Hepes buffer. The glands were cleaned of any adhering tissues under a dissecting microscope, and used either for preparation of dispersed cells or homogenized for the protein kinase assay.

2.2. *Preparation of dispersed parathyroid cells and cyclic AMP assay*

The dispersed cells were prepared essentially as in [4] and incubated in Eagle's medium containing 20 mM Hepes, 1 mM $CaCl_2$ and 10^{-4} M isobutylxanthine at 35°C. At the end of the incubation, some of the cell-containing medium was extracted with 10% perchloric acid for cyclic AMP assay. The remaining incubation medium was centrifuged and soluble cyclic AMP-dependent protein kinase was obtained from the cell pellet. Cyclic AMP was assayed by radioimmunoassay after acetylation according to a modification [3,11] of the method in [12]. The bound antibody was separated from the free ligand by the use of *Staphylococcus aureus* according to [13].

2.3. *Preparation of extracts of parathyroid gland extracts*

Glands from 5–10 vitamin D-deficient chicks were homogenized in ice-cold 100 mM MES buffer containing 1 mM EGTA and 0.1% mercaptoethanol, at pH 6.5. The homogenate was centrifuged in 27 000 × *g* for 15 min. The supernatant was used for protein kinase assay.

2.4. *Protein kinase assay*

Soluble, cyclic AMP-dependent protein kinase was released as in [14] but using 5 mM Tris buffer containing 1 mM Mg-acetate and 0.5 mM theophylline, at pH 7.2. Protein kinase was assayed as in [15] by measuring the transfer of ^{32}P from [γ-^{32}P]ATP to proteins. The incubation mixture (final vol. 0.2 ml) contained 50 mM MES buffer, 10 mM Mg-acetate, 1 mM theophylline, 10 mM NaF, 0.3 mg histone IIA, 100 μM ATP (labeled with [γ-^{32}P]ATP 2×10^6 cpm/tube) and 1 mM EGTA (except for the experiment with different

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calcium concentrations), at pH 6.5. Incubations were carried out at 30°C for 10 min.

3. Results

The dependence of protein kinase on cyclic nucleotides was studied by the addition of cyclic AMP to extracts of chick parathyroid gland in the kinase assay mixture (table 1). With no exogenous cyclic nucleotide in the reaction mixture, the enzyme activity was 5.1 nmol . mg^{-1} . min^{-1}. Addition of cyclic AMP caused a concentration-dependent increase in the enzyme activity. Half-maximal activation was found with 5×10^{-8} M, whereas maximum activation was obtained with 5×10^{-6} M of the cyclic nucleotide. Cyclic GMP hardly modified the enzyme activity. However, 5×10^{-6} M of cyclic GMP-stimulated kinase activity (2-fold), probably by some cross-reactivity with the cyclic AMP-specific sites. Cyclic AMP-dependent kinases can often be activated by higher molar concentration of cyclic GMP [16].

The response of kinase activity to an increase in endogenous cyclic AMP was studied in parathyroid cells treated with cholera toxin. This toxin is known to elevate cyclic AMP concentration in many cellular preparations in which it was tested, including bovine parathyroid cells [5]. In these cells, cholera toxin also stimulated PTH release. The time course of cholera toxin stimulation of cyclic AMP accumulation in dispersed avian parathyroid cells, is shown in fig.1. No change in the cyclic AMP concentration was observed in control cells during the experiment. When cholera

Table 1
Effect of cyclic nucleotide concentration on protein kinase activity of avian parathyroid gland

Cyclic nucleotide concentration (M)	Protein kinase activity (nmol P incorp. mg protein^{-1} . min^{-1})	
	+ Cyclic AMP	+ Cyclic GMP
0	5.1 ± 0.69[a]	
5×10^{-10}	5.5 ± 0.72	5.3 ± 0.72
5×10^{-9}	6.6 ± 0.81	5.2 ± 0.75
5×10^{-8}	20.0 ± 1.2	6.0 ± 0.93
5×10^{-7}	26.3 ± 1.5	7.2 ± 0.90
5×10^{-6}	40.1 ± 3.0	11.0 ± 1.0

[a] Average of 5 observations ± SEM

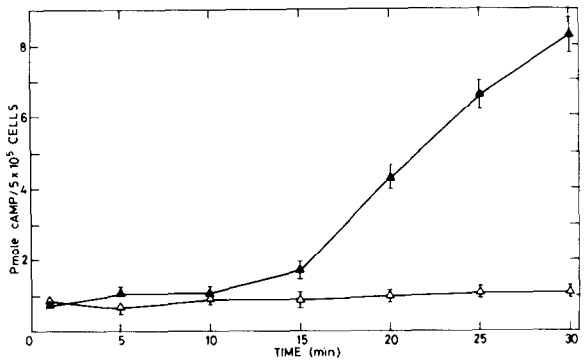

Fig.1. Effect of cholera toxin on cyclic AMP production by dispersed avian parathyroid cells. The cells were incubated in Eagle's medium with 20 mM Hepes buffer (pH 7.4) at 35°C with (▲) or without (△) 10 μg cholera toxin and the total cyclic AMP (cells + medium) was assayed as in section 2.

Table 2
Effect of cholera toxin on cyclic AMP concentration and protein kinase activity in dispersed parathyroid cells

	Cyclic AMP (fmol/2.5 × 10^5 cells)	Protein kinase activity (nmol P incorp. 2.5 × 10^6 cells^{-1} . min^{-1})	
		− Cyclic AMP	+ Cyclic AMP (5×10^{-6} M)
Control	483	6.8	16.6
+ cholera toxin (10 μg)	1640	12.0	15.4

Dispersed parathyroid cells were incubated in Eagle's medium for 30 min with or without cholera toxin. At the end of the incubation the cells were analyzed for endogenous cyclic AMP concentration and assayed for protein kinase activity with and without cyclic AMP in the assay mixture

Table 3
Effect of calcium concentration on protein kinase activity with or without cyclic AMP

Calcium concentration (mM)	Protein kinase activity (nmol P incorp. mg protein^{-1} . min^{-1})	
	– Cyclic AMP	+ Cyclic AMP (5×10^{-6} M)
0 + EGTA	11.2 ± 0.9[a]	40.3 ± 2.8
0.001	9.3 ± 0.8	49.2 ± 3.5
0.1	11.8 ± 0.9	48.3 ± 2.8
1.0	11.2 ± 0.9	38.9 ± 2.6
5.0	9.2 ± 0.7	22.3 ± 2.2
10.0	7.0 ± 1.0	12.8 ± 1.8

[a] Average of 5 observations ± SEM

toxin was added to the incubation medium, an almost linear rate of increase in total (cells + medium) cyclic AMP concentration was observed after a lag period of ~15 min. The magnitude of this lag period is ~1/3rd of that observed with dispersed bovine parathyroid cells [5]. After 30 min incubation in the presence of the toxin, an ~10-fold increase in cyclic AMP was observed. Table 2 shows the effect of cyclic AMP elevation due to cholera toxin on the cyclic AMP-dependent protein kinase activity.

Without cholera toxin, the kinase activity in the parathyroid cells was activated 2.4-fold by the addition of exogenous cyclic AMP to the reaction mixture. Cholera toxin caused a marked increase in the cyclic AMP concentration, apparently resulting in an increase in kinase activity. The response of these toxin-treated cells to exogenous cyclic AMP was slight.

The effect of different calcium concentrations on protein kinase activity with or without cyclic AMP is shown in table 3. In the absence of cyclic AMP, the kinase activity was hardly affected by changes in the calcium concentration from 1 nM–5 mM. Only a slight decrease was found with 10 mM calcium in the reaction mixture. On the other hand, the cyclic AMP-dependent protein kinase activity decreased as calcium in the reaction mixture increased from 0.1–10 mM. Calcium at 10 mM was sufficient to inhibit protein kinase activity to the level exhibited by the cells not stimulated by cyclic AMP. However, some calcium requirement of the system is suggested by the low kinase activity with EGTA, as compared with low concentration of calcium. The inhibition of kinase activity by calcium could be either direct or through the binding of ATP in the reaction mixture. The high

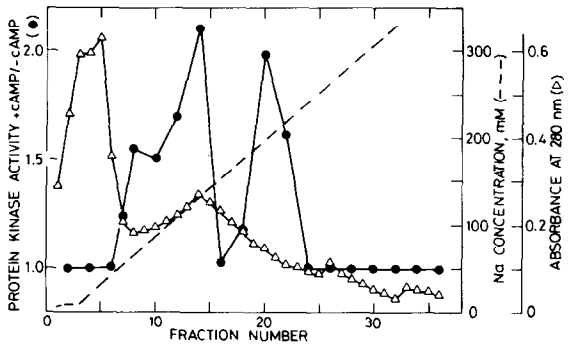

Fig.2. DEAE-Cellulose chromatography of protein kinases of avian parathyroid glands. Parathyroid glands were homogenized in 5 mM MES buffer containing 0.1% mercaptoethanol, 4 mM EDTA and 10^{-6} M phenylmethylsulfonylfluoride (PMSF) at pH 6.5. The homogenate was centrifuged for 15 min, 27 000 × *g*. The supernatant was applied on DEAE-cellulose column (12 × 1.2 cm) which was equilibrated with the same buffer but with 1 mM EDTA. Fractions of 6.5 ml were collected and assayed for protein kinase activity with or without 5×10^{-6} M of cyclic AMP.

Mg^{2+} concentration in the medium (10 mM), however argues against the latter possibility.

A first attempt at a characterization of the cyclic AMP-dependent protein kinase was made by applying a parathyroid gland extract to a DEAE-cellulose column which was eluted with a NaCl gradient (fig.2). Aliquots of the collected fractions were assayed for kinase activity with or without cyclic AMP. The results represent the ratio between the cyclic AMP-stimulated and the non-stimulated activity. At least two peaks of cyclic AMP-dependent kinase activity were apparent.

4. Discussion

A cyclic AMP-dependent protein kinase is demonstrated here in avian parathyroid tissue, similar to that of other tissue sources, including various endocrine glands [17]. In several mammalian tissues, cyclic AMP-dependent protein kinases have been classified as type I or II on the basis of their elution pattern from DEAE-cellulose column (review [18]). A very similar pattern is observed in fig.2, suggesting that the kinases of the avian parathyroid gland are similar in nature to the mammalian ones. The proportion of activities of type I to type II kinases varies over a wide range in different tissues. If one assumes that the classification of protein kinase in birds is similar to that proposed for mamma-

lian tissue, these results with avian parathyroid glands indicate that the activities of the two isoenzymes are similar in magnitude.

On the basis of stimulation experiments of bovine parathyroid cells with low calcium concentrations [4], β-agonists [4], cholera toxin [5] and dopamine [2], hormone secretion by parathyroid cells was shown to be proportional to cyclic AMP production [4], This suggests that PTH secretion is modulated by cellular cyclic AMP. Protein phosphorylation, known to follow cyclic AMP production, had not been demonstrated in parathyroid tissue. These present results add another link to the sequence of events resulting from stimulation of parathyroid cell and ending at hormone secretion.

Although stimulated by many agents, PTH secretion may be considered a feedback response to changes in ambient calcium concentration, which by itself is considered an important intracellular regulator, interacting with cyclic AMP. Using calcium ionophore A23187 [19], it was concluded that the effect of low calcium concentrations on parathyroid hormone secretion could not be explained completely on the basis of stimulation of the adenylate cyclase system resulting in elevation of cyclic AMP concentration. Phosphodiesterase may be considered as an additional site of calcium action on the parathyroid gland, as this enzyme system is known to be inhibited at high calcium concentrations [20]. These results show that calcium inhibits cyclic AMP-stimulated protein kinase activity in addition to inhibition of cyclic AMP production by the parathyroid cells [4], thus suggesting that protein kinase could be an additional regulatory site of calcium action in the parathyroid gland.

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