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EFFECT OF CALCIUM ION ON HORSE PARATHYROID GLAND ADENYL CYCLASE

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SUMMARY

Studies were carried out to characterize some of the properties of the adenyl cyclase from the horse parathyroid gland. The enzyme was stimulated by epinephrine (10^{-4} to 10^{-6} M) as well as by 10 mM NaF. Mg^{2+} at concentrations as high as 30 mM did not saturate the basal reaction, but there was a maximum for Mg^{2+} at 10 mM in the NaF-stimulated reaction. The apparent K_a for this cation was estimated to be 3–4 mM. Ca^{2+} inhibited the enzyme activity. Half-maximal inhibitions for the basal, NaF- and epinephrine-stimulated reaction in the presence of 10 mM Mg^{2+} were achieved at Ca^{2+} concentrations between 0.8 and 1.4 mM. Concentration effect relationships suggest that Ca^{2+} altered the maximal velocity V of the enzyme. The effect of F^- was to increase V for Mg^{2+} and it had no effect on the affinity for Mg^{2+}. Inorganic phosphate (10^{-2} to 10^{-4} M), KCl (3 to 30 mM), prostaglandin E_1 (0.4 to 4 μg/ml), GTP (10^{-4} to 10^{-6} M) and 1,2-di(2-aminoethoxy)ethane-*N*,*N*,*N'*,*N'*,-tetraacetic acid (EGTA) (10^{-3} to 10^{-4} M) had no effect on the basal enzyme activity. The hypothesis that the inhibitory action of Ca^{2+} on the parathyroid adenyl cyclase might have a physiological significance in the regulation of parathyroid hormone secretion or parathyroid metabolism is discussed.

INTRODUCTION

Cyclic adenosine 3',5'-monophosphate (cyclic AMP) mediates the actions of a number of hormones and is implicated in the secretion of certain peptide hormones[1,2]. Considerable evidence[3–6] has indicated that the major factor controlling parathyroid hormone secretion is the plasma level of calcium. The secretion *in vivo* is inversely related to the amount of Ca^{2+} in the blood passing through the gland. Ca^{2+} is therefore the specific inhibitory signal of the parathyroid. If cyclic AMP is the intracellular compound through which the rate of parathyroid secretion is regulated, the activity of adenyl cyclase from the gland may be affected by Ca^{2+}.

Abbreviations: ACTH, adrenocorticotropin; EGTA, 1,2-di(2-aminoethoxy)ethane-*N*,*N*,-*N'*,*N'*-tetraacetic acid.

The purpose of the present work is to ascertain the presence of adenyl cyclase activity in the parathyroid gland, to define some characteristics of this enzyme, and to investigate its response to Ca^{2+} and Mg^{2+} concentration.

MATERIAL AND METHODS

Cyclic [^{3}H]AMP [7] (ammonium salt; 12.7 mCi/mg) and [α-^{32}P]ATP (0.6 to 1.5 Ci/mmole) were purchased from New England Nuclear (Boston) and the Radiochemical Centre (Amersham), respectively. [α-^{32}P]ATP was purified from an unidentified radioimpurity by column chromatography on Dowex 50W-X4 as described by Taunton *et al.*[8]. It was stored at −20 °C for 2–3 weeks. Dowex 50W-X4, 200–400 mesh (H^+ form) was obtained from Bio-Rad Laboratories (Richmond, Calif.). Crystalline bovine serum albumin and α-epinephrine were purchased from Calbiochem, phosphocreatine and creatine kinase (75 units/mg) from Sigma, 1,2-di(2-aminoethoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) from B.D.H. Chemicals, Ltd (Poole, England), and caffeine and NaF from E. Merck (Darmstadt). All other reagents were commercial preparations.

Horse parathyroid glands were obtained from a local abattoir at the time of killing and transported to the laboratory in an ice-cold 0.25 M sucrose buffered by 5 mM Tris–HCl (pH 7.4). The glands were cleansed, sliced and homogenized in a glass tube using a motor-driven teflon pestle. The homogenates were filtered through three layers of cotton gauze before use. The identity of tissue preparations was checked by light microscopy. Protein of the parathyroid homogenates was measured by the method of Lowry *et al.*[9], using bovine albumin as a standard.

Adenyl cyclase activity was assayed by measuring the conversion of [α-^{32}P]ATP to cyclic [^{32}P]AMP in the presence of Mg^{2+}, caffeine and an ATP-regenerating system. The standard system contained 50 mM Tris–HCl (pH 7.4), 10 mM $MgCl_2$, 3.2 mM [α-^{32}P]ATP ($4 \cdot 10^6$–$6 \cdot 10^6$ cpm), 10 mM caffeine, 0.1% bovine albumin, 10 mM phosphocreatine and 4.5 units creatine kinase in a final volume of 0.2 ml. After addition of all components except enzyme, the assay tubes were preincubated at 37 °C for 35 s and the reaction was initiated by adding 0.1 ml of parathyroid homogenate (incubation time 5 min, temperature 37 °C). The reaction was terminated by boiling for 3.5 min after addition of 0.1 ml of 33.3 mM ATP and 12.5 mM cyclic [^{3}H]AMP (0.1 μCi). The method for separating cyclic [^{32}P]AMP was adapted from Krishna *et al.*[7]. After cooling in ice, the reaction mixture was diluted with 0.25 ml of water and centrifuged at 1500 × *g* for 10 min to remove heat-denatured protein. The supernatant fluid was applied to a 0.6 cm × 4.5 cm column of Dowex 50W-X4 in the hydrogen form and the cyclic AMP was eluted with water, between the 5th and 8th ml of effluent. To this 4-ml fraction were added 0.1 ml 0.25 M $ZnSO_4$ and 0.1 ml 0.25 M BaOH. The mixture was agitated and centrifuged at 1500 × *g* for 10 min. Two further precipitations and centrifugations were carried out. 2 ml of the resulting supernatant fluid were added to 15 ml of Bray's solution[10] and counted in a Nuclear Chicago scintillation spectrometer (Mark II). An internal standard was used for quench correction. Recovery of cyclic AMP, as estimated with cyclic [^{3}H]AMP, was between 60 and 75%. The amount of cyclic AMP formed during the incubation was calculated from the specific activity of [α-^{32}P]ATP in the reaction mixture and the amount of cyclic [^{32}P]AMP recovered *minus* the reaction blank. Blank estimates were carried out by using either no enzyme

or boiled enzyme; both gave essentially the same result. Reaction blank was less than 0.003% of the radioactivity added in ATP. The purity of the cyclic AMP isolated from Dowex 50W-X4 was established by thin layer chromatography on polyethyleneimine-cellulose[11]. For the evaluation of cyclic AMP degradation, cyclic [^{3}H]AMP 2.5 mM (0.2 Ci/mole), but not [^{32}P]ATP, was added to an assay system exactly identical to the system used for measuring adenyl cyclase. [^{3}H]ATP, [^{3}H]AMP and cyclic [^{3}H]-AMP were separated according to Krishna *et al.*[7] and their radioactivity measured as described before. All experiments were carried out at least in duplicates.

RESULTS

With parathyroid homogenate of a low protein concentration (0.27 mg) cyclic AMP accumulated progressively during 35 min of incubation both in the control and in the fluoride-treated samples (Fig. 1). The accumulation appeared to be linear with respect to the time of incubation only during the first 5 min in the control, but the time curve for the NaF-stimulated reaction was almost linear during the first 10 min. With a higher concentration of enzyme (1.0 mg protein), the amount of cyclic AMP detected after 35 min incubation was less than that of 5 or 15 min incubation. The amount of cyclic AMP produced in 5 min was directly proportional to enzyme concentration when the content of parathyroid protein in the incubation medium was 1 mg or lower. On this basis, most experiments were carried out with a 5-min incubation time and with a protein concentration of 0.2–0.6 mg in 0.2 ml of incubation medium. Depending on the preparation, NaF at the concentration of 10 mM produced a 70 to 240% increase over the basal levels.

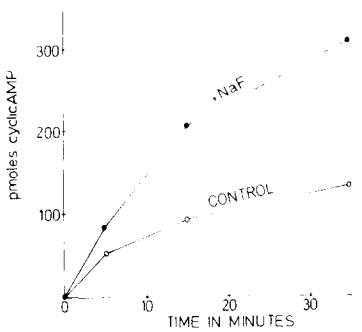

Fig. 1. Time course of the basal (○—○) and NaF-stimulated (●—●) adenyl cyclase activity. Fluoride was added at a final concentration of 10 mM. Each sample contained horse parathyroid homogenate (0.27 mg protein). Incubation conditions were described under Materials and Methods.

In an experiment to examine the stability of the enzyme, the parathyroid homogenate was kept at 4 °C and assayed at various intervals. The relative activity 0, 15, 30 and 45 min after homogenization was 100%, 93%, 89% and 81%, respectively. Because of this significant instability, fresh preparations were used for each experiment and additional control experiments were run when the time between homogenization and assay became higher than 15 min.

With ATP fixed at 3.2 mM, an increase in Mg^{2+} concentration caused a progressive increase in both unstimulated and NaF-stimulated activities (Fig. 2). The basal

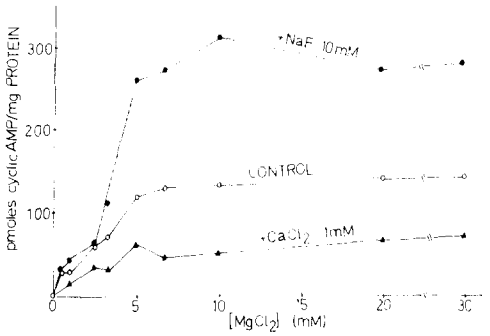

Fig. 2. Effect of Mg^{2+} concentration on the basal (○—○) and NaF-stimulated (●—●) adenyl cyclase activity and on the enzyme activity in the presence of 1 mM Ca^{2+} (▲—▲). ATP concentration, 3.2 mM.

reaction rate for the enzyme was already high at a concentration of 10 mM Mg^{2+} but was not maximal below 30 mM; in the presence of NaF, there was a maximum for Mg^{2+} at 10 mM. 10 mM of $MgCl_2$ was used for further study. Ca^{2+} at the concentration of 1 mM inhibited the adenyl cyclase at all concentrations of Mg^{2+}.

The effects of hormones and other substances on adenyl cyclase are summarized in Table I. Prostaglandin E_1 did not appear to have more effect on the enzyme than its solubilizing agent ethanol. Both epinephrine and NaF significantly stimulated the enzyme. The stimulatory effect of 10 mM NaF was greater than that of 10^{-4} M epine-

TABLE I

ACTION OF HORMONES AND CHEMICAL AGENTS ON ADENYL CYCLASE ACTIVITY OF HORSE PARATHYROID HOMOGENATE

All results are means of closely agreeing duplicates in one representative experiment. The basal activity of adenyl cyclase was 126.5 ± 5.6 pmoles/5 min per mg protein (mean ± standard error).

Agent	*Concentration*	*% of control*
NaF	10 mM	189
Prostaglandin E_1	0.4 μg/ml	109
	4 μg/ml	120
Ethanol	0.5 %	114
KH_2PO_4	0.1 mM	97
	0.3 mM	99
	1 mM	102
	3 mM	105
	10 mM	110
KCl	3 mM	97
	10 mM	110
	30 mM	95
$CaCl_2$	0.5 mM	82
	1.0 mM	54
EDTA	0.1 mM	107
	1 mM	103
GTP	10^{-6} M	113
	10^{-5} M	100
	10^{-4} M	108
Epinephrine	10^{-6} M	125
	10^{-5} M	135
	10^{-4} M	139

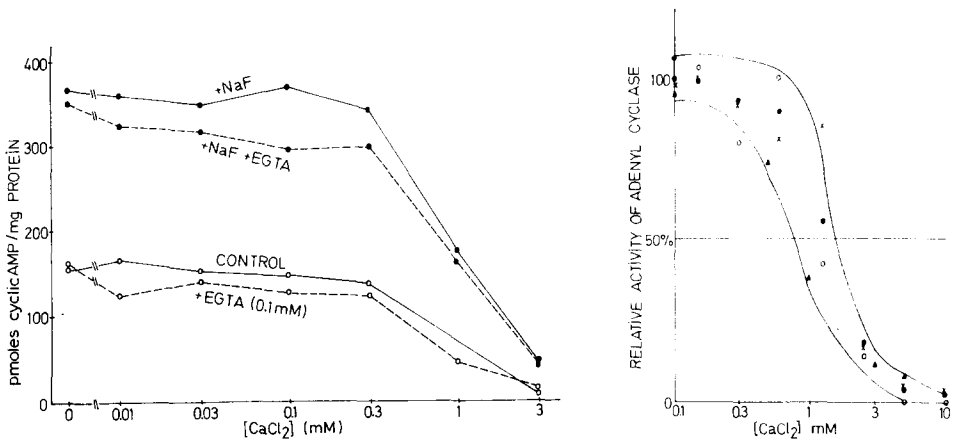

Fig. 3. Effect of Ca^{2+} and EGTA on the basal and NaF-stimulated adenyl cyclase activity. EGTA and NaF were added at the final concentrations of 0.1 mM and 10 mM, respectively. ○—○, basal activity; ○---○, basal activity *plus* EGTA; ●—●, NaF-stimulated activity; ●---●, NaF-stimulated activity *plus* EGTA.

Fig. 4. Effect of various concentrations of Ca^{2+} on the basal NaF- and epinephrine-stimulated parathyroid adenyl cyclase activity. In the presence of 10 mM $MgCl_2$: ○—○, control; ●—●, 10 mM NaF; ×—×, 0.1 mM epinephrine. In the presence of 5 mM $MgCl_2$: ▲—▲, control.

phrine. Inorganic phosphate, KCl, guanosine triphosphate (GTP) and EDTA seemed to have little effect on the enzyme. Ca^{2+} inhibited the enzyme at concentrations of both 0.5 and 1 mM.

Inhibition of the enzyme by Ca^{2+} was examined in a variety of conditions. Ca^{2+} inhibited the basal and NaF-stimulated enzyme (Fig. 3). With 0.3 mM $CaCl_2$ only a slight inhibition was observed, but the activity was reduced to approximately 50% by 1 mM $CaCl_2$. No stimulatory effect was observed at lower concentrations of Ca^{2+}. Combination of 0.1 mM EGTA and various concentrations of Ca^{2+} did not greatly alter the basal and NaF-stimulated enzyme activities. The enzyme activity curves of the basal and NaF- or epinephrine-stimulated enzyme in the presence of Ca^{2+} appeared sigmoidal (Fig. 4). Half maximal inhibitions for the basal, NaF- and epinephrine-stimulated adenyl cyclase were estimated to lie between 0.9 and 1.5 mM. Decreasing the Mg^{2+} concentration from 10 to 5 mM seemed to shift the inhibition curve to the left, *i.e.*, to increase the sensitivity to Ca^{2+}. The nature of Ca^{2+} inhibition was explored in experiments in which the effect of this cation on the Mg^{2+} saturation curve was examined. Apparent K_a values for Mg^{2+} were of the order of 3–4 mM. As in other systems, NaF increased the maximal velocity V of the enzyme without affecting the K_a value for Mg^{2+}. The V values for the basal and NaF-stimulated reactions in one of these experiments were estimated as 33 and 59 pmoles per min per mg protein, respectively. Ca^{2+} decreased the V of the enzyme but the scatter of the data did not allow a clear demonstration of an increased K_a for Mg^{2+}.

Cyclic [^{3}H]AMP degradation and [^{3}H]ATP formation from cyclic AMP were minor after 5 min of incubation (20% and 15% of added cyclic [^{3}H]AMP respectively) but larger after 15 min (50% and 30% respectively). No effect of Ca^{2+} (3 mM) was observed at 5 min, but after 15 min the medium contained somewhat less cyclic [^{3}H]-

AMP in the presence of Ca^{2+} ($43 \pm 1\%$ *vs* $57 \pm 1\%$ of total cyclic [^{3}H]AMP added respectively).

DISCUSSION

The present study has demonstrated that the horse parathyroid gland possesses an adenyl cyclase activity which is responsive to both NaF and epinephrine but not to prostaglandin E_1. Many properties of the enzyme from this gland seem to be similar to those found for the enzyme from other tissues. The parathyroid adenyl cyclase requires Mg^{2+} for its activity. The basal enzyme reaction was not saturated even by 30 mM Mg^{2+}, but there was maximum for Mg^{2+} at 10 mM in the NaF-stimulated reaction (Fig. 2). The K_a for Mg^{2+} is as in cardiac tissue of the order of 3 mM[11]. The cation stimulated the enzyme activity at concentrations far in excess of that required for Mg^{2+}–ATP complexing. Since the substrate for the enzyme is likely to be an Mg^{2+}–ATP complex[8,12,13], it appears that as in other tissues, the enzyme can bind Mg^{2+} in addition to the catalytic site at a second site (possibly allosteric) that influences its catalytic activity. The affinity of this second site for Mg^{2+} would be 30–20 times less than the affinity of the Mg^{2+}–ATP complex for the catalytic site[12]. The possibility was considered by Drummond and Duncan[12] that Mg^{2+} might serve in physiological regulation of the enzyme *in vivo* by binding to this second site. The apparent K_a for this cation was estimated to be 3–4 mM in the parathyroid. As in other tissues, fluoride stimulated the enzyme activity, by increasing the V without modifying the apparent affinity for Mg^{2+} (refs 12, 13). Parathyroid adenyl cyclase was not activated by prostaglandin E_1, but it was enhanced by epinephrine as in many other tissues[2,14].

A calcium dependency has been reported for some hormone-stimulated adenyl cyclases. The complete removal of Ca^{2+} with a calcium chelator resulted in the specific loss of ACTH-stimulated activity in adrenal cortex[8,14–17] and in fat cells[13] and a loss of NaF-stimulated activity in calf brain[17] but did not decrease glucagon and epinephrine activity in the fat cells[13]. It was interpreted by Birnbaumer *et al.*[13] that ACTH, unlike glucagon and epinephrine, requires calcium for its interaction with its receptor. The adenyl cyclase from the parathyroid gland was not inhibited by EGTA, nor activated by calcium at low concentrations. Unlike adenyl cyclase in other tissues such as heart[12] and liver[18], parathyroid adenyl cyclase was not activated by EGTA.

The function of the parathyroid gland is to secrete parathyroid hormone when the level of blood Ca^{2+} falls. The signal to which the parathyroid responds is therefore the level of Ca^{2+}. If parathyroid adenyl cyclase were the receptor for this signal, one would expect: (1) The level of enzymatic activity to be high in the absence of Ca^{2+} (stimulated state) and low in the presence of Ca^{2+} (basal state); (2) The activity of the enzyme to vary over the same concentration range as other parameters of parathyroid function, such as secretion; (3) The enzyme to respond to the same agents as the tissue.

Parathyroid adenyl cyclase activity corresponded indeed in the absence of Ca^{2+} to activities in stimulated tissues and in the presence of Ca^{2+} to basal activities in such tissues. This action of Ca^{2+} cannot be explained by an activation of cyclic AMP hydrolysis, although such an effect is possible. There is a direct proportionality between parathyroid hormone secretion rate and plasma calcium in the range of plasma calcium from 6 to 12 mg per 100 ml[6]. The adenyl cyclase appears to be slightly more susceptible to calcium inhibition than the rate of secretion, but the calcium effects on

these two parameters may be comparable because only ionized calcium and not total calcium level regulates secretion. The only difference is that the enzyme activity curves in the presence of Ca^{2+} appear sigmoidal but this may approximate a linear relationship in the narrow range of plasma Ca^{2+} concentration.

It has also been reported that a low plasma magnesium level increases the level of circulating parathyroid hormone and a high magnesium level diminishes it[3-6]. The effect of Mg^{2+} on the parathyroid adenyl cyclase, however, is utterly different from that of Ca^{2+}. Essentially no inhibitory effect of Mg^{2+} on the enzyme was observed at concentrations as high as 10 mM, while relatively high concentrations of Mg^{2+} inhibit secretion. This discrepancy could perhaps be explained by the fact that if adenyl cyclase would be negatively regulated by Ca^{2+} and Mg^{2+} on the exterior of the plasma membrane, Mg^{2+} would, as in other tissues, be necessary for enzymatic activity, and might even allosterically activate it inside the cell. Inorganic phosphate plays no direct role in regulating parathyroid hormone[3-6], nor does it affect the adenyl cyclase activity as shown in the present study. The data obtained in the present study are thus compatible with the hypothesis that the Ca^{2+} signal acts on the parathyroid through the adenyl cyclase–cyclic AMP system, but do not prove it. Ca^{2+} also inhibits adenyl cyclase in other tissues: fat, heart, and adrenal tissues[12,13,16], but to a lesser extent. This inhibitory effect has not been adequately explained. Birnbaumer *et al.*[13] have suggested that calcium and magnesium compete for a putative allosteric site on the adenyl cyclase enzyme in the fat cell membrane. In the cardiac tissue[12] Ca^{2+} inhibition was competitive with respect to Mg^{2+}. In the present study, no competitive inhibition was shown in the parathyroid, which might indicate another mechanism of action of Ca^{2+}.

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