Journal of Supramolecular Structure 9:391–398 (1978)
Transmembrane Signaling 375–382

Effects of PTH and Ca^{2+} on Renal Adenyl Cyclase

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The effects of calcium ion on the adenylate cyclase system was studied in isolated, renal basal-lateral plasma membranes of the rat. Bovine parathyroid hormone (bPTH) and a guanyl triphosphate analogue, Gpp(NH)p were used to stimulate cyclase activity.

Under conditions of maximal stimulation, calcium ions inhibited cyclic adenosine monophosphate (cAMP) formation, the formation rate falling exponentially with the calcium concentration. Fifty percent inhibition of either bPTH- or Gpp(NH)p-stimulated activity was given by approximately 50 μM Ca^{++}. Also the Hill coefficient for the inhibition was close to unity in both cases. The concentration of bPTH giving half-maximal stimulation of cAMP formation (1.8×10^{-8} M) was unchanged by the presence of calcium.

These data suggest that calcium acts at some point other than the initial hormone-receptor interaction, presumably decreasing the catalytic efficiency of the enzymic moiety of the membrane complex.

Key words: parathyroid hormone, adenylate cyclase, calcium, guanylylimidodiphosphate

Hormones, guanylate nucleotides, and divalent cations all modulate adenylate cyclase activity in mammalian tissues. While the adenylate cyclase activity in a variety of systems is enhanced by guanosine triphosphate (GTP), hydrolysis-resistant GTP analogues such as guanylylimidodiphosphate (Gpp[NH]p), polypeptide hormones, and catecholamines, it is inhibited by calcium [1–8]. This is also true for parathyroid hormone-sensitive adenylate cyclase found in rat renal cortical plasma membranes [9, 10].

Many hormones whose action is to stimulate adenylate cyclase activity at the target cell plasma membrane have been reported to influence calcium entry into the cell as well [7]. Within the cell, the newly generated cyclic adenosine monophosphate (cAMP) acts to regulate the rates of certain biochemical processes, while simultaneously the calcium acts to limit the rising cAMP level. This limitation is expressed through both the stimulation of phosphodiesterase activity and the inhibition of adenylate cyclase activity [7].

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Received March 27, 1978; accepted August 8, 1978.

0091-7419/78/0903-0391$01.70

Since hormones and guanyl nucleotides appear to act synergistically, it is surmised that their respective enhancements of adenylate cyclase activity are enacted through related mechanisms. In studying the mode whereby calcium inhibits adeylate cyclase activity, it was therefore of interest to investigate whether calcium affected parathyroid hormone-stimulated and Gpp(NH)p-stimulated cyclase activities differently.

In this report basal-lateral plasma membranes were isolated from rat renal cortical tissue homogenates by differential centrifugation followed by free-flow electrophoresis. This membrane preparation was utilized to compare the kinetic parameters for calcium inhibition of Gpp(NH)p-stimulated and parathyroid hormone-stimulated adenylate cyclase activity.

MATERIALS AND METHODS

Basal-lateral plasma membranes from rat renal cortical homogenates were isolated by the method of Heidrich et al [11], as described previously [12]. They contained Na-K adenosine triphosphatase (ATPase) activity and minimal alkaline phosphatase activity. Adenylate cyclase activity was determined by measuring the conversion of [α-^{32}P]-ATP to [^{32}P]-cAMP, during incubation of the basal-lateral plasma membranes at 37°C for 15 min. The final reaction volume was 50 μl, comprised of the following: 5 μl basal-lateral membrane solution, 5 μl of 10 mM ATP containing 0.5–2.0 μCi of [^{32}P]-ATP, 5 μl of the appropriate agonist or antagonist, and 5 μl of the appropriate diluent, 5 μl of H_2O if necessary for the final volume, and 30 μl of buffer which consisted of 4.5 mM $MgCl_2$, 30 mM KCl, 9 mM theophylline, 3.3 mg/ml bovine serum albumen (BSA), 0.83 mM cysteine, 0.5 mg/ml creatine phosphokinase, 42 mM creatine phosphate, 21 μg/ml myokinase, and 50 mM Tris-HCl, pH 7.5. As described elsewhere [12] cAMP formation (Fig. 1) was linear

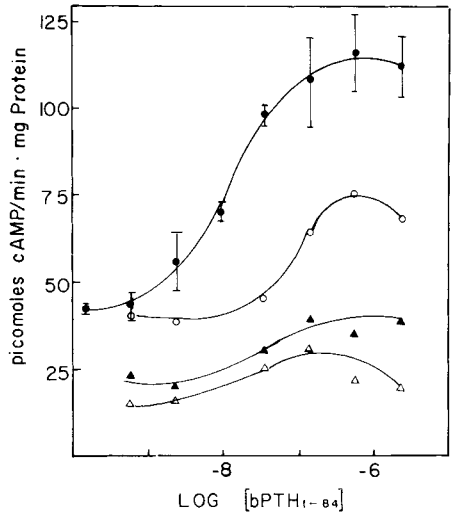

Fig. 1. Cyclic AMP formation as a function of $bPTH_{1-84}$ concentration. Adenylate cyclase activity was measured for 15 min at 37°C as described in Methods. Samples contained $bPTH_{1-84}$ at the molarity indicated on the abscissa and zero calcium (●—●), 0.15 mM calcium (○—○), 0.4 mM calcium (▲—▲), or 0.9 mM calcium (△—△). $CaCl_2$ was the last reagent added prior to the 37°C incubation period. Data points for control curve (0 mM $CaCl_2$) are average of triplicate determinations ± SEM; all other points are averages of duplicates.

during this time period and linear with the addition of membrane protein up to 20 μg; phosphodiesterase activity was negligible. (Points shown are averages of duplicate or triplicate determinations as specified.) Cyclic AMP was separated from other adenine nucleotides by the method (C) described by Salomon et al [13]. Ion exchange columns of Dowex 50 W were 6 cm × 0.4 cm, while the neutral alumina columns (Bio-Rad) were 2.5 cm × 0.4 cm. All columns were prepared fresh for each experiment and were not reused. All solutions of bovine parathyroid hormone ($bPTH_{1-84}$) or Gpp(NH)p were prepared fresh immediately prior to incubations with membranes. Parathyroid hormone was diluted in 0.01 N HCl containing 2 mg/ml BSA and 0.25×10^{-6} M mercaptoethanol using tubes precoated with cetyl alcohol to eliminate adsorptive losses [14]. All incubations were carried out in tubes precoated with cetyl alcohol. Protein was determined by the method of Lowry [15] using BSA as a standard.

Mathematical calculations were carried out as described previously [12]. Carboxymethylcellulose-purified $bPTH_{1-84}$, the activity of which was 3,000 Medical Research Council Units per milliligram, was generously provided by H. Keutman.

RESULTS

The log-dose response curve for cAMP formation as a function of $bPTH_{1-84}$ concentration revealed a typical sigmoid curve (Fig. 1). Fifty percent of the maximal cAMP formation occurred at a concentration of 1.8×10^{-8} M $bPTH_{1-84}$, while maximal activity occurred at or above 0.55×10^{-6} M $bPTH_{1-84}$. A Hill plot of these data (Fig. 2) revealed an excellent fit by linear regression analysis (correlation coefficient $r^2 = 0.98$) and a Hill coefficient n_H of 1.02 ± 0.07 (± SEM).

As has been previously reported [16], a decrease in cAMP formation was observed when calcium was present during the adenylate cyclase assay. As the concentration of calcium present during the incubation was increased, the maximum value for the $bPTH_{1-84}$-stimulated cAMP production became smaller, while the concentration of hormone which elicited one-half the respective maximum values did not change substantially (see Table I). At calcium concentrations of 400 μM or higher, virtually no stimulation of cAMP formation

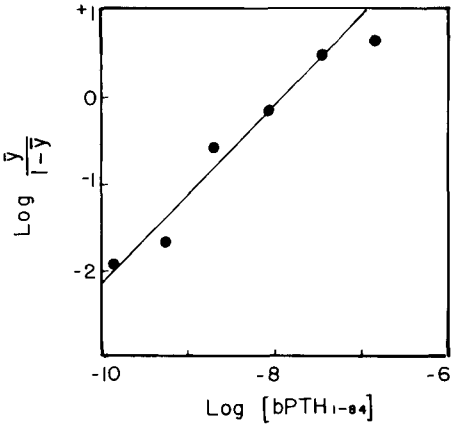

Fig. 2. Hill plot of data from parathyroid hormone-stimulated adenylate cyclase activity. Y is defined as the fraction of the maximal $bPTH_{1-84}$-stimulated cAMP formation elicited by a given concentration of $bPTH_{1-84}$. The slope of the line corresponds to the Hill coefficient n_H, which here was found to be 1.02 ± 0.07 (± SEM).

TABLE I. Quantitative Parameters: Inhibition of $bPTH_{1-84}$-Stimulated Adenylate Cyclase Activity by Calcium

Concn of calcium (mM)	cAMP production as % of control maximum		$bPTH_{1-84}$ concn[c] eliciting 50% of maximum
	Basal[a]	Maximum[b]	
0.00 (control)	37	100	2×10^{-8}M
0.10	40	74	1×10^{-8}M
0.15	34	66	5×10^{-8}M
0.40	19	34	3×10^{-8}M
0.90	13	26	2×10^{-8}M

[a]Basal level of cAMP production is that which occurs in the absence of $bPTH_{1-84}$, but in the presence of the indicated calcium concentration.
[b]Maximum level of cAMP production is that which occurs in the presence of 1 μM $bPTH_{1-84}$ and the indicated calcium concentration; 100% = 114×10^{-12} moles·min^{-1}·mg protein^{-1}.
[c]The hormone concentration necessary to elicit 50% of the maximum cAMP production observed for a given dose-response curve for which all samples contained the indicated calcium concentration.

by $bPTH_{1-84}$ was observed; considerable inhibition of the basal level of cAMP formation was seen as well (Fig. 1 and Table I).

The formation of cAMP in the presence of 1.0 μM $bPTH_{1-84}$ (a level which evokes maximal stimulation) and increasing amounts of calcium was measured. The amount of cAMP formed decreased exponentially as the calcium concentration rose (Fig. 3). A 50% decrease in $bPTH_{1-84}$-stimulated cAMP formation was caused by 60 μM calcium. This value for 50% inhibition differs from that obtained by Marcus and Aurbach [16], reported earlier (60 μM vs 1.6 mM). The nearly 30-fold increase in sensitivity to calcium by the present preparation may result from different methodologies used in the membrane preparations in the two studies.

No evidence was found that calcium inhibition occurred in a cooperative manner. The data for the inhibition of $bPTH_{1-84}$-stimulated adenylate cyclase activity yielded a Hill coefficient of -0.93 ± 0.13 and a correlation coefficient of 0.93 (Fig. 4). In this experiment 1 mM nonradioactive cAMP was present in all samples, to insure that the lowered levels of cAMP measured were a reflection of decreased synthesis of the cyclic nucleotide rather than increased destruction by phosphodiesterase. Repeating the experiment in the absence of nonradioactive cAMP yielded essentially identical results: a Hill coefficient of -0.95 ± 0.18, a correlation coefficient of 0.85, and 50% inhibition of cAMP formation by 60 μM calcium (data not shown).

Dose-response curves for $bPTH_{1-84}$ were carried out in the presence of 100 μM (data not shown) and 150 μM Ca (Fig. 1). When Ca was present at a concentration of 100 μM, the Hill coefficient was 0.98 ± 0.24 and the correlation coefficient was 0.85, while in the presence of 150 μM calcium, the Hill coefficient was 1.15 ± 0.30 and the correlation coefficient was 0.94. As the calcium concentration increases, the magnitude of cAMP formation becomes increasingly smaller, making the data increasingly difficult to analyze quantitatively. Nonetheless these Hill coefficients do not indicate that calcium changes the basic interaction of $bPTH_{1-84}$ with the receptor-adenylate cyclase system.

The effect of calcium on the Gpp(NH)p-activated state of the enzyme was also investigated. The characteristics of calcium inhibition of cAMP formation were found to be

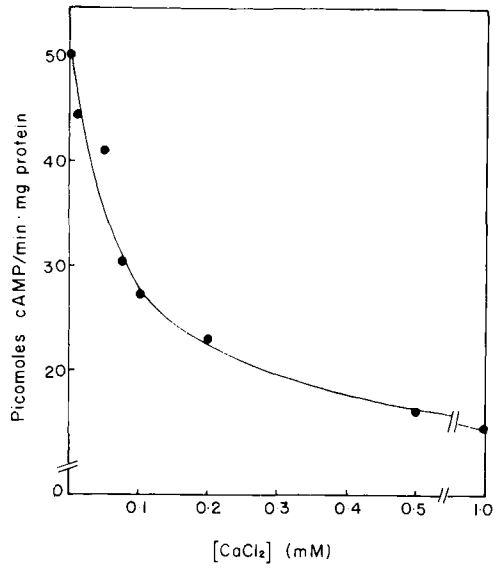

Fig. 3. Effect of increasing calcium concentrations on the adenylate cyclase activity stimulated by $bPTH_{1-84}$. All samples contained 1 μM $bPTH_{1-84}$, 1 mM nonradioactive cAMP, and the calcium concentration indicated on the abscissa. Cyclic AMP formation was measured as described in Methods. Each point is the average of triplicate determinations.

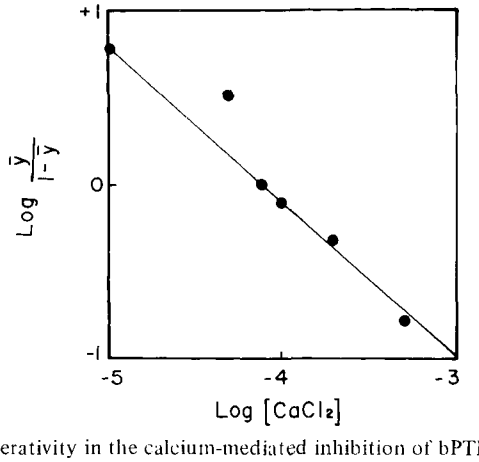

Fig. 4. Lack of cooperativity in the calcium-mediated inhibition of $bPTH_{1-84}$-stimulated adenylate cyclase activity. On a Hill plot the slope (± SEM) was -0.93 ± 0.13 and the correlation coefficient was 0.93.

similar whether $bPTH_{1-84}$ or Gpp(NH)p was used as the stimulating agent. Identical results were obtained if 1 mM cold cAMP was present in all reaction mixtures as well. The inhibition of Gpp(NH)p-stimulated formation also was an exponential process at calcium concentrations below 200 μM (Fig. 5). Fifty percent inhibition was achieved with 50 μM calcium, while the Hill coefficient was -1.01 ± 0.06 and the correlation coefficient was 0.92 (Fig. 6).

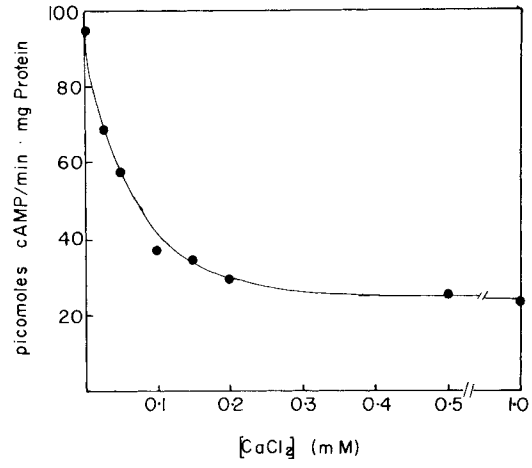

Fig. 5. Gpp(NH)p-activated adenylate cyclase activity as a function of calcium concentration. Immediately prior to incubation at 37°C, Gpp(NH)p was added to samples (final concentration = 10 μM) followed by addition of calcium solution at the appropriate concentration. Cyclic AMP formation was determined as described in Methods. Each point is the average of duplicate determinations.

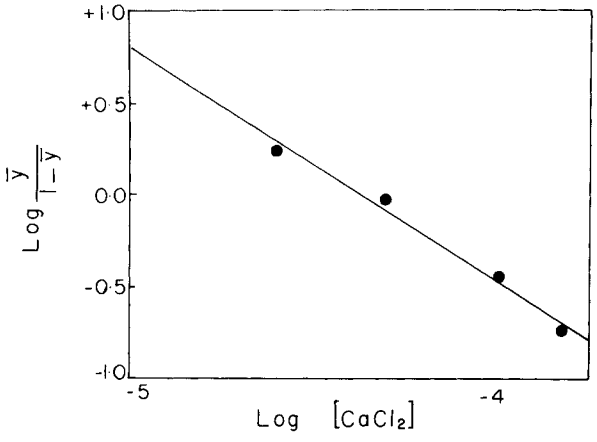

Fig. 6. Hill plot of calcium inhibition of Gpp(NH)p-stimulated cAMP formation. The slope of the line (n_H, the Hill coefficient), fit by linear regression analysis, is 1.01 ± 0.06. The correlation coefficient is 0.92.

DISCUSSION

A linear relationship was seen when the rate of cAMP formation (stimulated by Gpp(NH)p or $bPTH_{1-84}$) was plotted as a logarithmic function versus the calcium concentration present (not shown). This suggests that the inhibition of adenylate cyclase activity by calcium initially follows first-order kinetics with respect to calcium concentration, in much the same manner as an enzyme-catalyzed reaction follows first-order kinetics with regard to substrate concentration (under conditions where the free enzyme is present in excess of the substrate and the enzyme is noncooperative).

Taken together, the data suggest that calcium interacts with the receptor-adenylate cyclase system to decrease its catalytic efficiency. Similar phenomena have been reported in catecholamine-stimulated adenylate cyclase systems from turkey erythrocytes and frog

bladder epithelial cells, as well as in human polymorphonuclear leukocytes and in histamine-sensitive adenylate cyclase from hippocampus [2, 3, 8, 17, 18]. In the former cases, the derived Hill coefficients for the inhibition of adenylate cyclase activity were 2.0, suggesting a cluster of calcium ions, whereas, in agreement with this report, the Hill coefficient was 1.0 when leukocyte membranes were used. The closeness of the Hill coefficients to unity indicates that the calcium inhibitory action is mediated by a single calcium ion at each receptor-adenylate cyclase enzyme complex. Further, the agonist stimulating the adenylate cyclase activity is not critical to the calcium inhibitory effect since like effects are seen with both $bPTH_{1-84}$ and Gpp(NH)p.

Determination of the intracellular cytoplasmic levels of free calcium has remained a difficult task, but it is likely that they are normally below 1.0 μM in mammalian cells [19]. If this is true, the calcium-mediated inhibition, which here requires micromolar concentrations of calcium, could not occur under normal physiologic circumstances. However, it may well be that since the plasma membrane is involved in the binding, sequestering, and release of calcium, localized, elevated calcium concentrations could occur in proximity to the cell's periphery [20, 21]. This may be especially applicable to membranes derived from the basal-lateral surfaces from renal proximal tubular cells, reflecting the fact that parathyroid hormone alters renal ion transport and in particular enhances calcium retention by the kidney [22–24]. In addition, parathyroid hormone has been demonstrated to increase calcium influx into several cell types [25–27].

At the target cell plasma membrane some hormones accelerate the rates of calcium influx and cAMP synthesis [7, 19]. Alterations in intracellular cAMP and calcium levels then affect biochemical regulatory processes within the cell, including those that regulate the cAMP and calcium levels themselves. This negative feedback capacity of each upon the other thus limits the magnitude of changes. Thus it is quite possible that calcium inhibition of parathyroid hormone-sensitive adenylate cyclase activity is a physiologically operative negative feedback mechanism.

Other possible explanations for the observed phenomena must be ruled out, however. For instance, under the conditions employed, the binding of calcium by the membrane phospholipids would not be expected to exceed a small fraction of the calcium added [28]. Though both monovalent and divalent cations would be attracted to the anionic membrane surface, any tendency of calcium to be bound nonspecifically would be effectively counteracted by competition from the much higher concentrations of magnesium (2.7 mM) and potassium (20 mM). In addition, calcium-induced vesicle formation from phospholipid membranes requires millimolar concentrations of calcium [29]. For these reasons, the possibility that calcium inhibition is mediated by a generalized calcium-induced change in membrane fluidity is considered remote.

Calcium is not likely to exert its action by limiting substrate (ie, Mg^{++}-ATP) availability. Calcium inhibition has been observed in the absence of an ATP-regenerating system [8, 19]. Further, significant displacement of magnesium by calcium from the Mg^{++}-ATP complex would not occur under the conditions used here, since 1) calcium is bound less avidly to ATP than magnesium, and 2) the magnesium concentration exceeds the calcium concentration [5, 19].

Recently a calcium-dependent regulator protein was isolated which dramatically increased the activity of phosphodiesterase, the enzyme which hydrolyzes cAMP [30]. To eliminate the possibility that the calcium effect seen in these experiments could be exerted by such a protein, the experiments were repeated in the presence of nonradioactive cAMP. Under these conditions cAMP hydrolysis should be restricted to the nonradioactive cAMP, which is present in much greater abundance than the $[^{32}P]$-cAMP generated from $[^{32}P]$-

ATP during the experiments. No differences were observed. Theophylline (6 mM) was present in all reaction mixtures, which also makes unlikely the possibility of phosphodiesterase activation.

In these experiments the sensitivity of response for both PTH stimulation and calcium inhibition of adneylate cyclase activity exceeded that observed previously [16, 31, 32]. A partial explanation of this may be the purity of the hormone preparation and the great care that was taken to eliminate problems arising from hormone adsorption and denaturation. In addition, major differences exist between the methodologies used for the membrane preparations in these experiments and those used previously. Preparation of the basal-lateral plasma membrane fraction by free-flow electrophoresis avoids contamination from other subcellular organelles, especially from the luminal, or brush border, cell surfaces which contain extensive proteolytic activity.

ACKNOWLEDGMENTS

This work was supported in part by National Institutes of Health grants AM-17074 and 1F32-DE05119 and in part by the US Department of Energy contract No. E476-C02-3490 and has been assigned Report No. UR-3490-1362.

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