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Distribution of Parathyroid Hormone-Stimulated Adenylate Cyclase in Plasma Membranes of Cells of the Kidney Cortex*

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Summary. Free flow electrophoresis was employed to separate renal cortical plasma membranes into luminal (brush border microvilli) and contraluminal (basal-lateral membrane) fractions. During the separation adenylate cyclase activity was found to parallel the activity of Na^+-K^+-activated ATPase, an enzyme which is present in contraluminal but not in luminal membranes. In the basal-lateral membrane fraction the specific activities of adenylate cyclase and Na^+-K^+-activated ATPase were 4.4 and 4.6 times greater, respectively, than in the brush border fraction.

The adenylate cyclase of the basal-lateral membrane fraction was specifically stimulated by parathyroid hormone which maximally increased enzyme activity eightfold. The biologically active (1–34) peptide fragment of parathyroid hormone produced a 350% increase in adenylate cyclase activity. In contrast, calcitonin, epinephrine and vasopressin maximally stimulated the enzyme by only 55, 35 and 30%, respectively.

These results indicate that adenylate cyclase, specifically stimulated by parathyroid hormone, is distributed preferentially in the contraluminal region of the plasma membrane of renal cortical epithelial cells.

The plasma membrane of the proximal tubular epithelial cell is morphologically specialized, consisting of numerous microvilli (brush border) at the luminal surface and of interdigitating processes (basal infoldings) at the contraluminal cell face. The morphological polarity of the cell membrane is paralleled by an enzymatic polarity as shown in recent studies which demonstrated that alkaline phosphatase is a component of the brush border while Na^+-K^+-activated ATPase is present in the basal-lateral membrane (Wilfong & Neville, 1970; Kinne, Schmitz & Kinne-

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Saffran, 1971; Schmidt & Dubach, 1971; Heidrich, Kinne, Kinne-Saffran & Hannig, 1972).

The functional differentiation of the proximal tubular epithelial cell membrane may also be reflected in the relationship between the initial interaction of parathyroid hormone with its target cell and the resulting regulation of phosphate, sodium, calcium and glucose reabsorption (Talmage & Kraintz, 1954; Samiy, Hirsch & Ramsay, 1965; Agus, Puschett, Senesky & Goldberg, 1971; Agus, Gardner, Beck & Goldberg, 1973). The physiological actions of parathyroid hormone on the kidney have been shown to be mediated through the activation of adenylate cyclase (Chase & Aurbach, 1967, 1968; Melson, Chase & Aurbach, 1970; Marcus & Aurbach, 1971). It has been suggested that this parathyroid hormone-sensitive enzyme, a component of plasma membrane (Forte, 1972; Marx, Fedak & Aurbach, 1972*a*; Shlatz & Schwartz, 1972), may be localized in the contraluminal region (Wilfong & Neville, 1970; Marx *et al.*, 1972*a*). In order to evaluate this hypothesis and to develop additional evidence pertinent to the site of the hormone-receptor interaction, we have studied the distribution of the parathyroid hormone-sensitive adenylate cyclase system in the renal cortical epithelial cell membrane using the method of free flow electrophoresis.

Experimental Procedures

Materials

[α-^{32}P]ATP (5 to 9 Ci/mmole) and cyclic [^{3}H]AMP (22 Ci/mmole) were purchased from New England Nuclear. ATP, cyclic AMP[1], AMP, Tris and EDTA were obtained from Sigma Chemicals. Creatine phosphate, creatine kinase, myokinase and bovine serum albumin were obtained from Boehringer Mannheim. Arginine-vasopressin (407 USP units per mg), prepared by solid phase synthesis, was obtained from Dr. R. Walter of the University of Illinois Medical Center, and bovine parathyroid hormone was obtained from Dr. H. Rasmussen of the University of Pennsylvania. Synthetic (bovine) parathyroid hormone (1–34) tetratriacontapeptide (3100 I.U. per mg) was purchased from Beckman Instruments. Synthetic human calcitonin was a gift from CIBA and L-epinephrine (hydrogen-tartrate) was obtained from Serva Biochemicals. Polygram CEL 300 PEI thin-layer plates were purchased from Brinkman Instruments. Instagel scintillation fluid was obtained from Packard Instrument Company. All other chemicals were reagent grade.

Methods

Preparation of Membrane Fractions. Partially purified plasma membranes from rat kidney cortex were prepared by differential centrifugation in isotonic sucrose medium as previously

1 Adenosine 3′,5′-monophosphate.

described (Pockrandt-Hemstedt, Schmitz, Kinne-Saffran & Kinne, 1972). Utilizing an FF4 free-flow electrophoresis apparatus developed by Hannig (1968), the plasma membranes of the renal cortical cells were separated into luminal (brush border microvilli) and contraluminal (basal-lateral membrane) fractions as monitored by enzymatic analysis (Heidrich *et al.*, 1972). The brush border fraction consisted of microvilli and possessed a high specific activity of alkaline phosphatase. The fraction derived from the basal-lateral plasma membrane contained a high specific activity of Na^+-K^+-activated ATPase and consisted of large sheets with trilaminar structure and occasional junctional complexes.

The electrophoresis buffer contained 8.5 mM acetic acid, 8.5 mM triethanolamine and 280 mM sucrose adjusted to pH 7.4 with 2 N NaOH. The buffer for the electrode vessels consisted of 100 mM triethanolamine and 100 mM acetic acid adjusted to pH 7.4 with 2 N NaOH. The conditions during the electrophoresis were the following: 90 ± 9 V/cm, 85 mA, electrophoresis buffer flow of 2 ml per hour per fraction and a temperature of 5 °C.

All assay procedures were carried out on freshly isolated membranes.

Adenylate Cyclase Assay. Adenylate cyclase activity was measured by a modification of the method described by Bär and Hechter (1969). The incubation medium contained 1 mM [α-^{32}P]ATP (0.1–0.2 μCi), 40 mM Tris-HCl buffer, pH 7.5, 4 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM cyclic AMP, 0.1% bovine serum albumin, 25 mM creatine phosphate, 0.3 mg per ml of creatine kinase, 12.5 μg per ml of myokinase and 30 to 60 μg membrane protein in a total volume of 50 μl. The samples were incubated for 10 min at 37 °C and the reaction terminated by the addition of 20 μl of a solution of 10 mM ATP, 10 mM AMP and 10 mM cyclic AMP, followed by boiling for 3 min. Cyclic [^{32}P]AMP was separated by chromatography on CEL 300 PEI thin-layer plates in 0.3 M LiCl. Radioactivity was measured in a Packard liquid scintillation spectrometer utilizing Packard Instagel scintillation fluid.

Phosphodiesterase Assay. Cyclic nucleotide phosphodiesterase activity was determined by measuring the appearance of [^{3}H]AMP and [^{3}H]adenosine from cyclic [^{3}H]AMP. The incubation medium consisted of 0.5 mM cyclic [^{3}H]AMP (0.05 μCi), 40 mM Tris-HCl buffer, pH 7.5, 4 mM $MgCl_2$, 0.1 mM EDTA and 30 to 60 μg membrane protein. The final incubation volume was 50 μl. The incubation and chromatographic procedures were the same as those described for the adenylate cyclase assay.

Alkaline Phosphatase Assay. Alkaline phosphatase activity was measured in the presence of 0.5% bovine serum albumin using the Merkotest® system.

Na^+-K^+-activated ATPase Assay. Na^+-K^+-activated ATPase was determined as previously described (Györy & Kinne, 1971).

Protein Determination. Determinations were carried out after precipitation of the protein with ice-cold 10% trichloroacetic acid according to the procedure of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin as a standard.

Results

Characteristics of Plasma Membrane Preparation

The membrane preparation used in the present study contained enzymes characteristic of the proximal tubular plasma membrane (Kinne *et al.*, 1971; Schmidt & Dubach, 1971; Heidrich *et al.*, 1972). As compared to the cortical homogenate, the specific activities of Na^+-K^+-activated ATPase and alkaline phosphatase were enriched approximately five- and

sevenfold, respectively, in the plasma membranes. Morphologically, the preparation consisted of microvilli and large, open sheets of trilaminar membranes possessing occasional junctional complexes. There was only minor contamination by other cellular organelles as judged morphologically as well as by the low specific activities of mitochondrial, endoplasmic reticular and lysosomal marker enzymes.

The plasma membrane preparation was found to contain both basal and parathyroid hormone-stimulated adenylate cyclase activity. In preliminary studies, it was observed that even when the creatine kinase-creatine phosphate, ATP-regenerating system was present in the incubation mixture, approximately 35% of the ATP was degraded to AMP during a 10 min period. With the addition of myokinase, the amount of ATP broken down was reduced to 11% and the basal and parathyroid hormone-stimulated adenylate cyclase activities were significantly increased. Utilizing the latter assay conditions, the importance of substrate availability was further demonstrated by studying the effect of ATP concentration upon reaction rate (Table 1). Within the concentration range employed, both the basal and hormone-stimulated adenylate cyclase activities were proportional to the concentration of ATP.

Adenylate cyclase activity in the absence and presence of parathyroid hormone was also examined both as a function of plasma membrane concentration (Fig. 1) and as a function of time (Fig. 2). The nonlinearity of cyclic AMP production at high membrane concentrations and with long periods of incubation may involve such factors as membrane aggregation, enzyme inactivation or even a residual inadequacy of the ATP-regenerating system.

Table 1. Effect of ATP concentration on adenylate cyclase activity of plasma membranes

ATP concentration	Adenylate cyclase activity	
	Basal	PTH
(mM)	(pmoles cAMP/min/mg)	
0.1	3.7	8.1
0.5	17.1	64.2
1.0	25.6	107.8

Unfractionated plasma membranes were incubated with various concentrations of ATP. Parathyroid hormone was present at a concentration of 1 μM. Results represent the mean of triplicate determinations using the same membrane preparation.

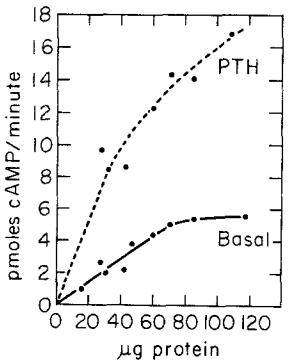

Fig. 1. Cyclic AMP production as a function of the concentration of unfractionated plasma membranes. Parathyroid hormone was present at a final concentration of 1 μM. Each point represents the mean of triplicate determinations in each of two separate preparations

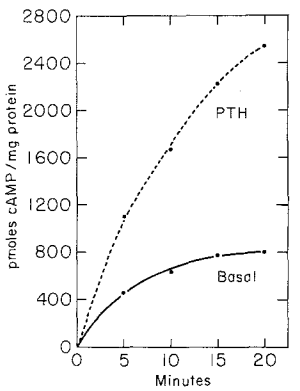

Fig. 2. Time course of adenylate cyclase activity in unfractionated plasma membranes. Parathyroid hormone was present at a final concentration of 1 μM. Each point represents the mean of triplicate determinations in each of two separate preparations

Phosphodiesterase activity associated with the plasma membrane preparation did not interfere with the determination of adenylate cyclase activity. In the presence of 100 μg of membrane protein less than 0.5% of the cyclic AMP in the incubation mixture was degraded in a 10 min period. Under the conditions of this experiment, all of the degraded nucleotide was found to be in the form of adenosine, indicating that the AMP formed by phosphodiesterase had been further converted to adenosine by 5′-nucleotidase, which we had previously shown to be a component of the brush border microvilli (Kinne *et al.*, 1971).

Localization of Adenylate Cyclase in the Basal Infoldings

Using free flow electrophoresis to separate the plasma membranes into luminal and contraluminal fractions, it was found that the adenylate cyclase specifically stimulated by parathyroid hormone followed the same general distribution pattern as Na^+-K^+-activated ATPase (Fig. 3), and that the maximum specific activities for both enzymes coincided in fraction 25. Following electrophoretic separation the recovery of parathyroid hormone-stimulated adenylate cyclase amounted to 30% and the recovery of Na^+-K^+-activated ATPase amounted to 47%. In those fractions which contained brush border microvilli as characterized by alkaline phosphatase activity, there was no significant adenylate cyclase activity. The distribution of basal adenylate cyclase activity followed the same pattern as the distribution of parathyroid hormone-stimulated adenylate cyclase activity after fractionation by free flow electrophoresis. Fig.4 compares the specific activities of adenylate cyclase, Na^+-K^+-activated ATPase, and alkaline phosphatase present in the unfractionated plasma membranes, the basal-lateral membrane fraction and the brush border membrane fraction. The ratios of enzyme activity in the basal-lateral membrane fraction to enzyme activity in the unfractionated plasma membranes were 1.1 and 1.4 for adenylate cyclase and Na^+-K^+-activated ATPase, respectively, but only 0.2 for alkaline phosphatase. The basal adenylate cyclase activity, the parathyroid hormone-stimulated adenylate cyclase activity and the Na^+-K^+-activated ATPase activity in the contraluminal membrane fraction were 4.4, 5.3 and 4.6 times greater, respectively, than in the luminal membrane fraction. The similarity of these enzyme activity ratios suggests

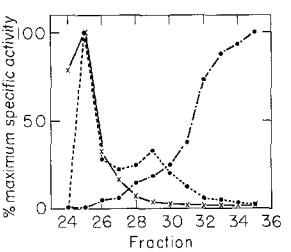

Fig. 3. Distribution of parathyroid hormone-sensitive adenylate cyclase (×——×), Na^+-K^+-activated ATPase (●----●), and alkaline phosphatase (●·····●) following free flow electrophoresis of renal cortical plasma membranes. Parathyroid hormone was present at a concentration of 1 μM. The maximal specific activity of parathyroid hormone stimulated adenylate cyclase was 1532 pmoles/hr × mg protein, of Na^+-K^+-activated ATPase 73.3 μmoles/hr × mg protein and of alkaline phosphatase 110.1 μmoles/hr × mg protein

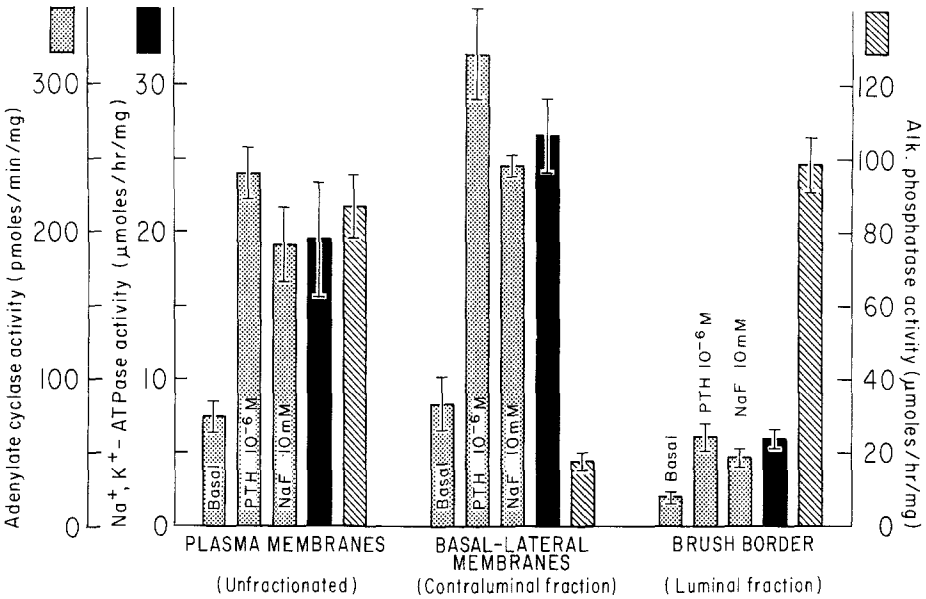

Fig. 4. Specific activities of adenylate cyclase, Na^+-K^+-activated ATPase and alkaline phosphatase in unfractionated plasma membranes, basal-lateral membranes (frations 20–26 in Fig. 3) and brush border membranes (fractions 33–39 in Fig. 3). Results are the mean ± SE of triplicate determinations using five separate preparations

that the low activities of adenylate cyclase (basal, parathyroid hormone-stimulated) and Na^+-K^+-activated ATPase found in the brush border fraction is a reflection of a minor "overlapping" contamination with membrane fragments derived from the region of the tight junction (where the contraluminal and luminal membranes meet) rather than a reflection of the presence of these enzymes in the brush border membrane *per se*.

Hormonal Stimulation of Adenylate Cyclase Activity

The stimulatory effect of several hormones upon the adenylate cyclase activity of the unfractionated, luminal and contraluminal membranes was measured as a function of hormone concentration over a range of 1 nM to 10 μM, as shown in Fig. 5. Parathyroid hormone produced by far the largest stimulation of adenylate cyclase activity of any agent tested; in the basal-lateral membranes, the maximal stimulation evoked by parathyroid hormone was approximately 8 times the basal activity. The maximal stimulation of enzyme activity by both epinephrine and arginine-vasopres-

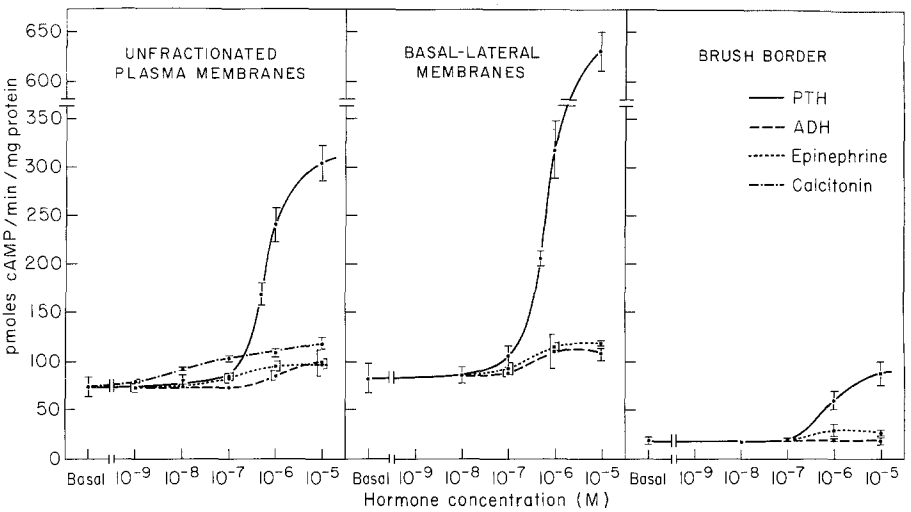

Fig. 5. Effects of hormones on adenylate cyclase activity in unfractionated plasma membranes, basal-lateral membranes and brush border membranes. Each point represents the mean ±SE of triplicate determinations using five separate preparations

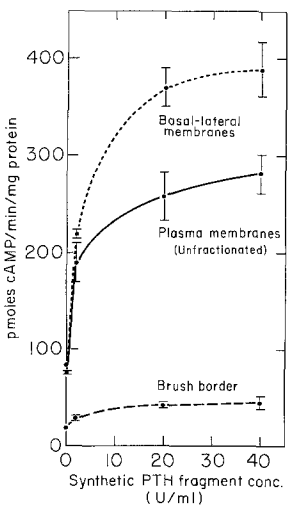

Fig. 6. Effect of synthetic parathyroid hormone (1–34) peptide on adenylate cyclase activity in unfractionated plasma membranes, basal-lateral membranes and brush border membranes. Each point represents the mean ±SE of triplicate determinations using four separate preparations

sin was only 30 to 35% above the basal level or less than 4% of the maximal stimulation produced by parathyroid hormone. As tested in the unfractionated plasma membranes, calcitonin maximally increased the adenylate cyclase activity by 58% at a concentration of 10 μM.

Since the biologically active (1–34) peptide fragment of parathyroid hormone was available, its effects on adenylate cyclase activity were also examined, as shown in Fig. 6. In the basal-lateral membranes the peptide maximally stimulated enzyme activity by 350% above basal levels; half-maximal stimulation was obtained at a concentration of approximately 2 Units per ml.

Stimulation of Adenylate Cyclase Activity by NaF

As illustrated in Fig. 7, fluoride stimulated the adenylate cyclase of the basal-lateral membranes maximally at a concentration of 10 mM, increasing the activity of the enzyme by 195% above basal levels. It should

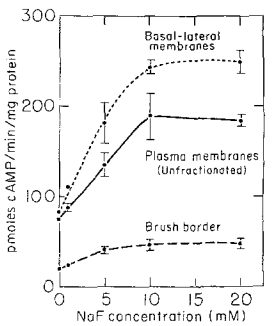

Fig. 7. Effect of varying concentrations of NaF on adenylate cyclase activity in unfractionated plasma membranes, basal-lateral membranes and brush border membranes. Each point represents the mean ± SE of triplicate determinations using four separate preparations

Table 2. Effect of NaF concentration on the conversion of ATP to AMP by the plasma membranes

NaF concentration (mM)	AMP formed (nmoles/min/mg)	% decrease
None	8.48	
1.0	8.04	5.2
5.0	4.13	51.3
10.0	1.20	85.8

Unfractionated plasma membranes were incubated with various concentrations of NaF as described in Experimental Procedures. The [^{32}P]AMP formed was separated by thin-layer chromatography and the radioactivity measured by liquid scintillation counting. Results represent the mean of triplicate determinations with each of two membrane preparations.

also be noted that the maximal NaF stimulation of adenylate cyclase activity was less than the maximal stimulation effected by parathyroid hormone. When NaF was presented to membranes that were already maximally stimulated by parathyroid hormone, there was no significant increment in activity.

In assaying adenylate cyclase activity as a function of NaF concentration it was noted that there was concomitant decrease in the amount of ATP degraded to AMP, as shown in Table 2; this observation suggests that, in addition to a direct effect on adenylate cyclase, NaF may also exert an indirect effect on enzyme activity by increasing the available level of substrate, perhaps by inhibiting an ATP pyrophosphohydrolase, which catalyzes the cleavage of ATP to AMP and PP_i. Such an enzyme has been demonstrated in plasma membranes isolated from rat liver (Ray, 1970).

Discussion

The specialized transport function of the proximal tubular epithelial cell is reflected in the differences in morphology and enzymatic composition of its luminal and contraluminal plasma membranes. Histochemical studies of the proximal tubule suggested that alkaline phosphatase is localized in the brush border (Molbert, Duspiva & Von Deimling, 1960), while Na^+-K^+-activated ATPase is present in the basal-lateral membranes (Ericsson & Trump, 1969). Subsequently, this suggestion was confirmed by separation of contraluminal and luminal membranes by microdissection of tubular cells (Schmidt & Dubach, 1971) and by electrophoresis of isolated plasma membranes (Kinne *et al.*, 1971; Heidrich *et al.*, 1972).

Wilfong and Neville (1970) purified a brush border fraction from rat kidney cortex which was characterized by a 15-fold increase in alkaline phosphatase activity and a twofold enrichment in Na^+-K^+-activated ATPase and adenylate cyclase activities as compared to the starting homogenate. The properties of this preparation further suggested that certain enzymes may be specifically localized in distinct membrane regions and that adenylate cyclase and Na^+-K^+-activated ATPase may be concentrated in the same area, presumably the contraluminal cell surface. However, the twofold enrichment in Na^+-K^+-activated ATPase and adenylate cyclase activities in the brush border preparation indicated either that the localization of these enzymes was not exclusively contraluminal or

that the brush border preparation was considerably contaminated by components of the contraluminal membrane.

Marx *et al.* (1972*a*) also concluded that Na^+-K^+-activated ATPase and adenylate cyclase were localized in contraluminal membranes of renal cortical cells; however, their data did not show a parallel enrichment of these enzymes (there was a sevenfold increase in adenylate cyclase activity in their membrane fraction relative to the crude homogenate but only a 2.7-fold increase in Na^+-K^+-activated ATPase activity).

In the present work utilizing free flow electrophoresis to effect a more complete separation of contraluminal and luminal membranes, it was found that parathyroid hormone-sensitive adenylate cyclase and Na^+-K^+-activated ATPase exhibited not only a parallel enrichment in the contraluminal membrane fraction but also a parallel decrease in the brush border fraction. This indicates that both enzymes are present in the same membrane, i.e., the basal-lateral membrane. Furthermore, although it is possible that the contraluminal membrane fraction may be contaminated in some degree with another PTH-cyclase containing entity that coelectrophoreses with the basal-lateral plasma membrane, it is more likely that parathyroid hormone-stimulated adenylate cyclase is exclusively localized in the contraluminal component of the proximal tubular epithelial cell envelope in the intact cell *in vivo*. In any case, the results do demonstrate unambiguously a preferential distribution of this enzyme in the isolated contraluminal membrane fraction.

The degree and specificity of the response to parathyroid hormone of the adenylate cyclase of the basal-lateral membranes is consistent with earlier studies which characterized the adenylate cyclase in isolated renal tubules (Melson *et al.*, 1970) and in renal cortical plasma membranes (Forte, 1972; Shlatz & Schwartz, 1972; Marx *et al.*, 1972*a*); parathyroid hormone increased the enzyme activity eightfold at maximal concentrations. The minor stimulation by vasopressin corresponds with the previously delineated anatomical localization of the parathyroid hormone- and antidiuretic hormone-sensitive adenylate cyclases in the cortex and medulla, respectively (Chase & Aurbach, 1968; Melson *et al.*, 1970; Marcus & Aurbach, 1971; Forte, 1972; Shlatz & Schwartz, 1972). In addition, the striking response to parathyroid hormone—which contrasts sharply with the relative lack of response to antidiuretic hormone—suggests that the membranes were derived primarily from the proximal tubule (the major renal site of action for parathyroid hormone) rather than the late distal tubule or cortical collecting duct (the target sites for antidiur-

etic hormone). The slight increment in enzyme activity observed in the presence of calcitonin may indicate a minor contamination of the preparation by membranes derived from the cortico-medullary junction since it has been suggested that the calcitonin-sensitive adenylate cyclase is localized in this zone (Marx *et al.*, 1972*a*; Marx, Woodward & Aurbach, 1972*b*).

Recent micropuncture studies of rat renal proximal tubules have demonstrated that parathyroid hormone produced a marked decrease in isotonic volume flux and glucose transport when injected into the peritubular capillaries as compared to the minor effect produced when the hormone was applied from the lumen [2]. These findings support the conclusion that parathyroid hormone-stimulated adenylate cyclase is preferentially, if not exclusively, distributed in the basal-lateral membranes. Furthermore they indicate that, although parathyroid hormone has access to the luminal as well as the contraluminal cell surface *in vivo*, its action is initiated at the contraluminal cell surface. Similarly, in isolated collecting tubules, vasopressin was found to be effective in increasing net water absorption along an osmotic gradient only when presented to the serosal surface (Grantham & Burg, 1966). These findings suggest that hormone receptors as well as enzymes may be functionally localized in specific regions of the plasma membrane of tubular epithelial cells.

[^{125}I]parathyroid hormone has been shown to bind specifically to plasma membranes isolated from bovine renal cortex (Sutcliffe, Martin, Eisman & Pilczyk, 1973; DiBella, Dousa, Miller & Arnaud, 1974). In further characterizing the relationship between the hormone receptor and adenylate cyclase, it is of interest to determine whether specific parathyroid hormone binding is limited to the contraluminal plasma membrane. In preliminary experiments, we have observed that high affinity parathyroid hormone binding sites are present both in the luminal and contraluminal membrane fractions; this finding indicates either that the hormone binding to the luminal surface is nonspecific or that both cell surfaces contain the receptor component of the cyclase system, but that only in the contraluminal membrane is the receptor functionally coupled to the enzyme. Similarly, a recent attempt to localize calcitonin receptors in renal membranes and to correlate their distribution with the polarization of adenylate cyclase was not definitive since high affinity sites were found in both the brush border and contraluminal membrane preparations (Marx, Woodward, Aurbach, Glossman & Keutmann, 1973).

2 Personal communication from Dr. K. Baumann.

Since the parathyroid hormone-sensitive adenylate cyclase has been shown to be a predominant component of the basal-lateral membrane, it is necessary to elucidate the mechanism by which hormone activation at the contraluminal surface leads to the characteristic parathyroid hormone-induced alterations of solute reabsorption in the proximal tubule. In the following paper (Kinne, Shlatz, Kinne-Saffran & Schwartz, 1975) evidence is presented which suggests that the cyclic AMP-dependent step in the mechanism of parathyroid hormone action takes place at the brush border of the cell.

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