J. Membrane Biol. 24, 145–159 (1975)

Distribution of Membrane-Bound Cyclic AMP-Dependent Protein Kinase in Plasma Membranes of Cells of the Kidney Cortex

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Received 24 April 1975; revised 8 July 1975

Summary. Renal cortical plasma membranes were separated by free flow electrophoresis into luminal (brush border microvilli) and contraluminal (basal-lateral membrane) fractions. These membranes were found to contain an intrinsic, self-phosphorylating system which consists of a cyclic AMP-dependent protein kinase, a phosphoprotein phosphatase and the substrate(s) of these enzymes. The kinase, but not the phosphatase, was stimulated by cyclic AMP; maximal (1.7-fold) stimulation was effected at a cyclic AMP concentration of 0.1 μM. The degree of phosphorylation of the brush borders was six times greater than that of the basal-lateral membranes in the absence of cyclic AMP and 2.3-fold greater in the presence of cyclic AMP. This preferential phosphorylation of the luminal membrane by membrane-associated protein kinase(s) may play a role in the parathyroid hormone-mediated alterations of solute reabsorption in the proximal tubule.

It is well established that parathyroid hormone regulates the reabsorption of phosphate in the proximal tubule of the kidney (Samiy, Hirsch & Ramsay, 1965; Frick, 1969; Agus, Puschett, Senesky & Goldberg, 1971; Gekle, 1971; Agus, Gardner, Beck & Goldberg, 1973; Beck & Goldberg, 1973) and that this action is mediated by the stimulation of a cortical adenylate cyclase (Chase & Aurbach, 1968; Melson, Chase & Aurbach, 1970; Shlatz & Schwartz, 1972). The morphological and enzymatic polarity of the proximal tubular epithelial cell, which provides the basis for transepithelial transport processes, may also be a factor in the mechanism of parathyroid hormone action. Recent studies concerning the localization of the parathyroid hormone-sensitive adenylate cyclase (Shlatz, Schwartz, Kinne-Saffran & Kinne, 1975) have shown that this enzyme is present predominantly, if not exclusively, in the contraluminal region of the proximal tubular epithelial cell membrane. Since the effects of adenosine 3′,5′-monophosphate may be mediated through regulation of the activity of

cyclic AMP-dependent protein kinases, it is of interest to determine whether such an enzyme is a component of the proximal tubular epithelial plasma membrane and whether it is also localized at one pole of the cell. Recently, plasma membranes isolated from the hog kidney cortex (Forte, Chao, Walkenbach & Byington, 1972) were shown to contain a cyclic AMP-dependent protein kinase. The properties of this enzyme are similar to other intrinsic protein kinases found in brain, synaptosomal and microsomal membranes (Weller & Rodnight, 1970, 1971, 1973*a, b*; Johnson, Maeno & Greengard, 1971; Maeno, Johnson & Greengard, 1971; Ueda, Maeno & Greengard, 1973), adenohypophyseal secretory granules (Labrie, Lemaire, Poirier, Pelletier & Boucher, 1971; Lemaire, Pelletier & Labrie, 1971), anterior pituitary gland microsomes (Lemay, Deschenes, Lemaire, Poirier, Poulin & Labrie, 1974), cardiac sarcoplasmic reticulum (Wray, Gray & Olsson, 1973), and erythrocyte membranes (Guthrow, Allen & Rasmussen, 1972; Rubin, Erlichman & Rosen, 1972; Roses & Appel, 1973; Rubin & Rosen, 1973).

The experiments reported in this paper demonstrate that preparations of luminal membranes and, to a lesser extent, contraluminal membranes contain an intrinsic cyclic AMP-dependent protein kinase as well as one or more of its substrates. The findings, in conjunction with recent physiological data, suggest that regulation of the level of phosphorylation of the luminal membrane may be an intermediate or final step in the chain of events which constitutes the action of parathyroid hormone on the proximal tubule.

Experimental Procedures

Materials

[γ-^{32}P]ATP (12 to 25 Ci/mole) and [^{14}C]ATP (10 mCi/mg) were purchased from New England Nuclear. ATP, cyclic AMP, Tris, EGTA and histone (calf thymus, Type II) were obtained from Sigma Chemicals. Bovine serum albumin was purchased from Boehringer Mannheim. Type HA, 0.45 μ filters were purchased from the Millipore Corp. Instagel scintillation fluid was obtained from Packard Instrument Co. 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 described (Heidrich, Kinne, Kinne-Saffran & Hannig, 1972). Utilizing an FF4 free-flow electrophoresis apparatus developed by Hannig (1968), the plasma membranes of the tubular

epithelial cells were separated into luminal (brush border microvilli) and contraluminal (basal-lateral membrane) fractions as monitored by alkaline phosphatase and Na^+-K^+-activated ATPase activities, respectively (Heidrich *et al.*, 1972; Shlatz *et al.*, 1975).

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).

Preparation of Cytosolic Protein Kinase. Soluble, cyclic AMP-dependent protein kinase was prepared from rat kidney cortex following the method described by Kuo, Krueger, Sanes and Greengard (1970). The purification procedure was carried through the $(NH_4)_2SO_4$ precipitation and the dialyzed enzyme solution was rapidly frozen utilizing a dry ice-acetone mixture. The activity of the enzyme preparation was monitored by its ability to phosphorylate histone (40 μg) substituted for the membrane protein in the standard assay. Basal activity ranged from 74 to 114 pmoles of ^{32}P incorporated per min per mg histone per mg "enzyme" protein; activity in the presence of 10^{-6} M cyclic AMP ranged from 130 to 172 pmoles per min per mg histone per mg "enzyme" protein.

Protein Kinase Assay. Intrinsic cyclic AMP-dependent protein kinase activity was determined by a modification of the procedure of Kuo *et al.* (1970). The standard assay mixture contained 50 mM sodium acetate, pH 6.5, 25 μM [γ-^{32}P]ATP (0.3 μCi), 10 mM $MgCl_2$, 0.3 mM EGTA, 20 mM NaF, 2 mM theophylline, and 20 to 40 μg membrane protein in a final volume of 0.2 ml. Cyclic AMP was added to give a final concentration in the assay medium ranging from 0.01 to 5 μM.

The samples were incubated at 30 °C for 5 min and the reaction was terminated by the addition of 2 ml of ice-cold 5% trichloroacetic acid. The samples were filtered through HA 0.45 μ Millipore filters and washed three times with 5 ml of 5% TCA. The filters were dried and then counted in a Packard Tri-Carb liquid scintillation spectrometer utilizing Instagel scintillation fluid. All assays were performed in triplicate.

To measure additional phosphorylation of the membrane occurring in the presence of cytosolic protein kinase, 110 μg of the soluble enzyme preparation was added to the incubation mixture.

Determination of [^{14}C]ATP-Binding. The amount of ATP which binds to the isolated membrane fractions under the conditions used for phosphorylation was measured by substituting [^{14}C]ATP (0.1 μCi) for [γ-^{32}P]ATP in the standard incubation mixture for protein kinase activity. The incubation and filtration procedures were the same as those described in the protein kinase assay. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer.

Protein Determination. Protein determinations were carried out according to the procedure of Lowry, Rosebrough, Farr and Randall (1951) using bovine serum albumin as a standard.

Results

Protein Kinase and Phosphatase Activities in Plasma Membranes

Plasma membranes from rat kidney cortex were phosphorylated by an intrinsic protein kinase and an additional uptake of [^{32}P] phosphate (about 25%) was observed when renal cortical cytosolic protein kinase was included in the system (Fig. 1). Cyclic AMP increased the rate of

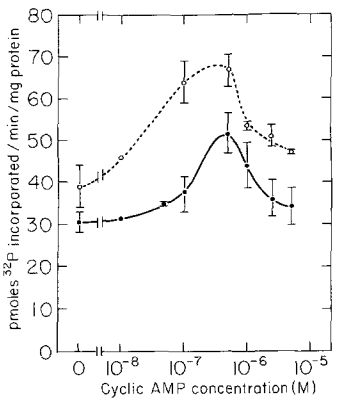

Fig. 1. Effect of cyclic AMP on the phosphorylation of unfractionated plasma membranes by an intrinsic protein kinase (●—●) and additionally in the presence of cytosolic protein kinase (○----○). Each point represents the mean ± SE of triplicate determinations in each of three separate preparations

incorporation of [^{32}P] phosphate both in the presence and in the absence of the cytosolic enzyme. Maximal intrinsic protein kinase activity was attained in the presence of 0.5 μM cyclic AMP; this maximal activity was 1.7 times greater than the activity obtained under basal conditions.

No binding of [^{14}C]ATP to the plasma membranes was detected either in the presence or in the absence of cyclic AMP, indicating that all of the ^{32}P incorporated into the membranes represented transfer of the terminal phosphate group of ATP to membrane components and not adsorption of the entire ATP molecule. None of the ^{32}P incorporated proved to be involved in the formation of a phosphorylated intermediate of Na^+-K^+-activated ATPase because neither potassium nor ouabain altered the degree of membrane phosphorylation (Table 1).

Table 1. Effect of potassium and ouabain on the phosphorylation of unfractionated plasma membranes

Addition	Protein kinase activity (pmoles/min/mg protein)
None	36.3
KCl	34.3
KCl + ouabain	39.3
Ouabain	33.8

KCl and ouabain were added to the incubation mixture at final concentrations of 20 and 2 mM, respectively. Results represent the mean of triplicate determinations using the same membrane preparation.

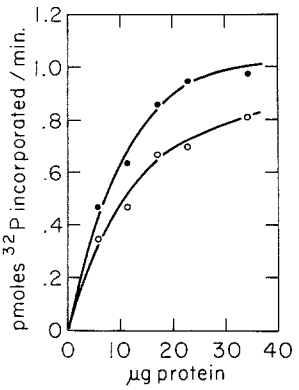

Fig. 2. Protein kinase activity as a function of the concentration of unfractionated plasma membrane in the absence (o—o) and in the presence (●—●) of 0.5 μM cyclic AMP

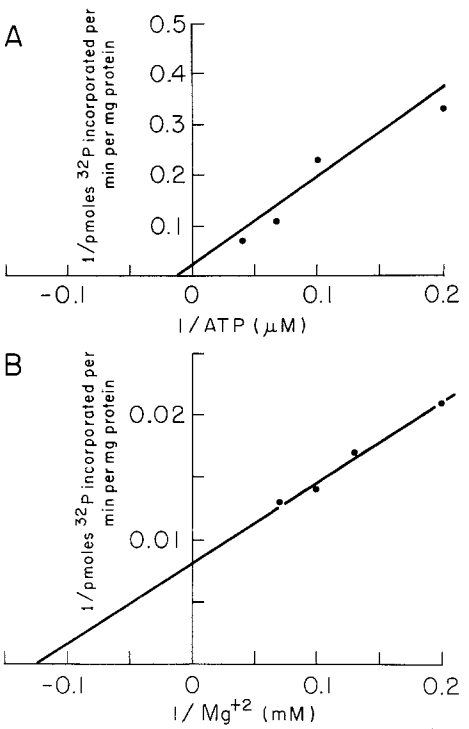

Fig. 3. Effects of varying concentrations (*A*) of ATP and (*B*) of Mg^{2+} on the intrinsic protein kinase activity of unfractionated plasma membranes

Protein kinase activity, both in the presence and absence of cyclic AMP, proved to be dependent on but not proportional to protein concentration as shown in Fig. 2. For this reason equivalent amounts of protein were used in all determinations. The dependence of enzyme activity on ATP and magnesium concentration is illustrated in Figs. 3*A* and 3*B*, respectively.

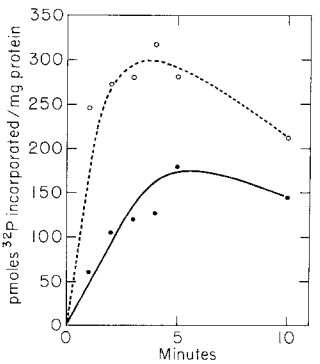

Fig. 4. Time course of phosphorylation of unfractionated plasma membranes by an intrinsic protein kinase (●——●) and additionally by cytosolic protein kinase (○----○)

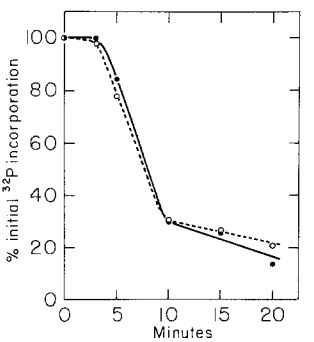

Fig. 5. Dephosphorylation of unfractionated plasma membranes in the absence (●——●) and in the presence (○----○) of 0.1 μM cyclic AMP. The membranes were phosphorylated as described in Experimental Procedures in the absence and in the presence of 1 μM cyclic AMP. At zero time, the samples were diluted 10-fold with buffer to minimize any further phosphorylation and incubation continued at 30 °C for the time periods indicated

The time course of ^{32}P-labeling of the plasma membranes is indicated in Fig. 4. Incorporation was observed during the initial 3–5 min of incubation after which the ^{32}P-content of the membranes decreased. The dephosphorylation process was studied in more detail under conditions which minimized protein kinase activity; approximately 70% of the [^{32}P] phosphate was lost from the membranes in a period of 10 min (Fig. 5). The rate of this phosphatase reaction was neither stimulated nor inhibited in the presence of 0.1 μM cyclic AMP.

Distribution of Protein Kinase in the Cell Envelope

When the renal plasma membranes were subjected to free-flow electrophoresis, brush border microvilli, characterized by alkaline phosphatase

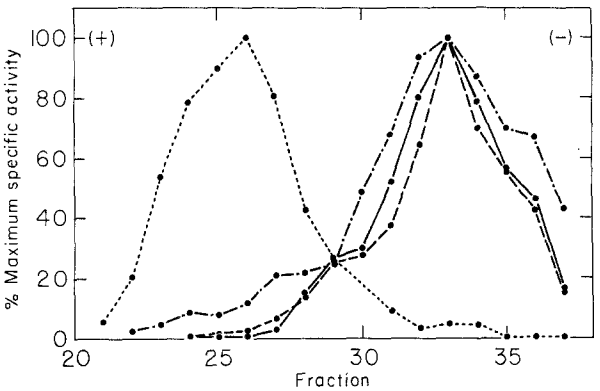

Fig. 6. Distribution of Na^+-K^+-activated ATPase (●----●), alkaline phosphatase (●–·–●), and intrinsic protein kinase in the absence (●——●) or in the presence (●– – –●) of 1 μM cyclic AMP following free flow electrophoresis of plasma membranes. The maximal specific activity of Na^+-K^+-activated ATPase amounted to 58.0 μmoles/hr × mg protein, of alkaline phosphatase 102.0 μmoles/hr × mg protein, of basal protein kinase 16.5 pmoles/min × mg protein and of cAMP-dependent protein kinase 30.0 pmoles/min × mg protein

activity, could be separated from the basal-lateral membranes which contain Na^+-K^+-activated ATPase activity. The basal and cyclic AMP-stimulated protein kinase activities migrated with the alkaline phosphatase activity (Fig. 6) suggesting that protein kinase is predominantly associated with the luminal plasma membrane.

The brush border fraction and the basal-lateral plasma membrane fraction exhibited marked differences in the specific activities of Na^+-K^+-activated ATPase, alkaline phosphatase and protein kinase as shown in Fig. 7. The activities of alkaline phosphatase and intrinsic protein kinase were both six times higher in the brush border microvilli than in the basal-lateral plasma membranes.

Fig. 8 shows the cyclic AMP-dependence of the intrinsic protein kinase activity of the brush borders as contrasted with that of the basal-lateral plasma membranes. The *relative* cyclic AMP dependence of the enzyme (i.e., the percent increase above basal activity) is greater in the basal-lateral membrane fraction than in the brush border fraction. However, at all concentrations of cyclic AMP, the *absolute* level of phosphorylation of the brush border fraction greatly exceeded that of the basal-lateral membrane fraction. It can also be seen in Fig. 8 that the inclusion of cytosolic protein kinase caused only an additional 25% increase in the maximal phosphorylation of the luminal membrane system.

It was possible that a selective enrichment of a protein kinase inhibitor in the contraluminal fraction might account for the difference in protein

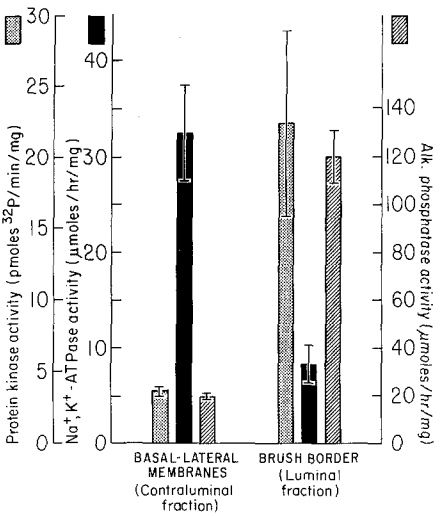

Fig. 7. Specific activities of intrinsic protein kinase, Na^+-K^+-activated ATPase and alkaline phosphatase in basal-lateral membranes and brush border microvilli. Results represent the mean ±SE of triplicate determinations in each of three separate preparations

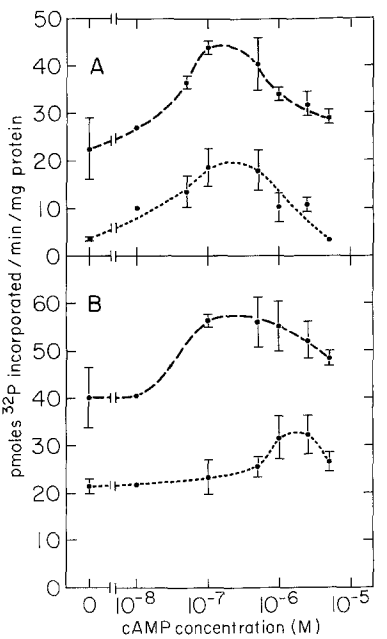

Fig. 8. (*A*) Effect of cyclic AMP on intrinsic protein kinase activity of brush border microvilli (●——●) and basal-lateral membranes (●----●). (*B*) Effect of cyclic AMP on the phosphorylation of brush border microvilli (●——●) and basal-lateral membranes (●----●) in the presence of cytosolic protein kinase. Results represent the mean ±SE of triplicate determinations using three separate preparations

kinase activity found in the two membrane fractions. This possibility was excluded by experiments in which the protein kinase activities of the brush border microvilli and basal-lateral membranes were first determined separately and then determined after combining equivalent amounts of each. Almost complete additivity (about 80%) was observed in these recombination experiments indicating either the absence or an equal distribution of inhibitory substances. The possibility that the Na^+-K^+-activated ATPase activity present in the contraluminal membranes limited their phosphorylation by reducing substrate availability was ruled out by including NaF in the incubation mixture at a concentration (20 mM) which completely inhibits Na^+-K^+-ATPase activity.

Attempts to Extract Membrane-Associated Protein Kinase

The cyclic AMP-dependent protein kinase activity of the plasma membrane could not be extracted with solutions of various ionic composition and ionic strength, nor by treatment with papain, which is known to remove the glycocalyx as well as loosely bound proteins (Pockrandt-Hemstedt, Schmitz, Kinne-Saffran & Kinne, 1972) (Table 2). These results support the view that the protein kinase activity associated with the membrane is indeed intrinsic and does not represent adsorbed cytosolic enzyme.

Table 2. Attempts to extract protein kinase from plasma membranes

Treatment	Protein kinase activity	
	Basal	Cyclic AMP
	(pmole/min/mg protein)	
None	8.0	13.2
NaCl	8.1	14.0
Papain	8.6	13.0

The plasma membranes were preincubated for 10 min at 37 °C with either 0.15 M NaCl or 50 μg papain per mg membrane protein. The membranes were sedimented by centrifugation for 15 min at 14,000 × *g*, resuspended in 0.1 M Tris buffer and the protein kinase activity determined as described in Experimental Procedures. Cyclic AMP was present at a final concentration of 1 μM. Results represent the mean of triplicate determinations using the same membrane preparation.

Discussion

The identification in renal tissue of cytosolic (Kuo & Greengard, 1969; Winickoff & Aurbach, 1970; Dousa, Sands & Hechter, 1972; Forte *et al.*,

1972; Wombacher, Reuter-Smerdka & Körber, 1973) and membrane-bound (Forte *et al.*, 1972; Shlatz, Kinne, Kinne-Saffran & Schwartz, 1973) cyclic AMP-dependent protein kinases led to the idea that these enzymes may play an effector role in the regulation of tubular transport processes by hormones which act through the adenylate cyclase-cyclic AMP system. Such a role implies that some component of the affected transport system serves as a substrate for the protein kinase and that this substrate is more active at one pole of the transporting cell. In the present study we have found that a membrane-bound cyclic AMP-dependent protein kinase is more active in the brush border fraction than in the basal-lateral membrane fraction. In the presence of cytosolic kinase we observed an approximately 25% increment in phosphorylation of membrane and possibly also of components of the cytosolic kinase preparation. Thus, the increment in phosphorylation may be due to action of cytosolic protein kinase on membrane-bound substrates or action of membrane-bound protein kinase on soluble substrates or both. In any case, since this increment is small, it is the intrinsic protein kinase which appears to be primarily responsible for maintaining the state of phosphorylation of the brush border.

Physiological data indicate that cyclic AMP acts on the renal tubule only from the luminal surface and that the luminal membrane is the ultimate site of action for parathyroid hormone (Kupfer & Kosofsky, 1970; Butlen & Jard, 1972; Baumann, Chan, Bode & Papavassiliou, 1974). Our data support this concept because (1) the absolute level of phosphorylation achieved in the presence of cyclic AMP is always higher in the brush border fraction than in the basal-lateral membrane fraction and (2) under the conditions which more closely mimic the physiological state (Fig. 8*B*, cytosolic components present) the cyclic AMP-dependence of the brush border protein kinase is much greater than the cyclic AMP dependence of the basal-lateral kinase. In addition, we have found repeatedly that brush border membranes tend to vesiculate to a greater extent than basal-lateral membranes; it is possible therefore that the difference between the cyclic AMP-dependent protein kinase activity of the brush border and the basal-lateral membranes *in vivo* is greater than that which we observed *in vitro*—because in the *in vitro* system both cyclic AMP and ATP would in all probability be excluded from contact with those active sites which are situated on the inner aspect of the vesicles.

It is possible that the regulatory subunit of the protein kinase of the brush border is more labile than that of the basal-lateral membranes thus accounting for the much higher basal kinase activity of the brush

border fraction and the relatively moderate increase in this activity in response to cyclic AMP.

It is also possible that the difference in self-phosphorylating activity of brush border as compared to basal-lateral membranes may reflect an uneven distribution of the kinase substrate rather than an asymmetric localization of the enzyme. Although we favor the view that membrane-bound kinase, membrane-bound phosphatase and their membrane-bound substrate(s) act as a functional unit to regulate the properties of the luminal membrane, we recognize that this is an assumption that must be supported by further evidence such as can be obtained, for example, by identifying the phosphorylated components in the different membranes under various experimental conditions.

The cyclic AMP-induced increase in uptake of [^{32}P] phosphate into the brush border membrane fraction appears to be due to the action of the nucleotide on protein kinase because cyclic AMP did not alter the phosphoprotein phosphatase activity of the plasma membranes. Similarly, a cyclic AMP-induced phosphorylation of the luminal membrane has been proposed to be the mechanism by which antidiuretic hormone alters permeability in the renal collecting duct (Dousa *et al.*, 1972; Schwartz, Shlatz, Kinne-Saffran & Kinne, 1974). In contrast, cyclic AMP has been shown to stimulate a membrane-associated phosphatase in the toad bladder (DeLorenzo, Walton, Curran & Greengard, 1973*a, b*) and it has been suggested that the increase in sodium transport induced by neurohypophyseal hormones in this tissue involves a dephosphorylation of a specific membrane component. The apparent differences in cyclic AMP mediation in the kidney and toad bladder may be explained by considering the possibility that the overall phosphorylation of the renal membrane may be masking a concurrent dephosphorylation of one or more specific phosphoproteins.

In principle, the asymmetric phosphorylation of the proximal tubular cell membrane might directly underlie all of the effects of parathyroid hormone on renal transport processes. However, such an alteration might account for the regulation of only one transport system, for example, the reabsorption of phosphate. Recent evidence (Agus *et al.*, 1973; Beck & Goldberg, 1973; Maesaka, Levitt & Abramson, 1973) has suggested that parathyroid hormone-induced changes in phosphate transport may be dissociated from the effects on calcium and sodium reabsorption. It is possible that these latter processes are regulated by a parathyroid hormone-induced increase in the cytosolic calcium concentration in the tubular cell (Rasmussen & Tenenhouse, 1968; Rasmussen, Goodman & Tenen-

house, 1972; Borle, 1973). The increase in calcium concentration might inhibit the Na^+-K^+-activated ATPase, resulting in a decrease in sodium reabsorption and, in turn, in an inhibition of transport processes either linked, e.g. calcium transport (Frick, Rumrich, Ullrich & Lassiter, 1965) or directly coupled, e.g. glucose transport (Frömter & Lüer, 1973; Kinne, Kinne-Saffran & Murer, 1973; Ullrich, Rumrich & Klöss, 1973) to sodium transport.

In conclusion, we are suggesting that the mechanism of action of parathyroid hormone on the proximal tubule involves an asymmetrical distribution of enzymes in the plasma membrane of the epithelial cell. Hormone action is initiated at the contraluminal membrane where the parathyroid hormone-sensitive adenylate cyclase system is localized (Shlatz *et al.*, 1975). The increase in intracellular cyclic AMP then serves to activate an intrinsic cyclic AMP-dependent protein kinase of the luminal membrane. Activation of this kinase leads to a preferential phosphorylation of one or more luminal membrane components which may alter the transport of phosphate and perhaps other solutes. The hormone-induced change in phosphorylation of the luminal membrane may be reversed by the action of a membrane-associated phosphoprotein phosphatase. This dephosphorylation process, in conjunction with the removal of parathyroid hormone from its receptor either by dissociation or degradation, terminates the hormone action.

This work was supported by USPHS grant AM-10080 of the National Institute of Arthritis, Metabolism and Digestive Diseases, the Life Sciences Foundation, Inc. and the Stella and Charles Guttman Foundation, Inc. L.J. S. is a recipient of USPHS Postdoctoral Fellowship AM-51842. The authors wish to thank Professor Karl J. Ullrich for his kind interest and valuable discussion.

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