Target Cell Polarity and Membrane Phosphorylation in Relation to the Mechanism of Action of Antidiuretic Hormone

(adenylate cyclase/protein kinase/renal collecting duct/luminal and contraluminal membranes)

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ABSTRACT The plasma membrane of the bovine renal collecting duct epithelial cell has been resolved into its apical (luminal) and basal-lateral (contraluminal) components by free flow electrophoresis. The contraluminal, but not the luminal, membrane was found to contain antidiuretic hormone-sensitive adenylate cyclase. The luminal membrane was found to contain a cyclic 3':5'-adenosine monophosphate-sensitive self-phosphorylating system consisting of a membrane-bound protein kinase and its membrane-bound substrate(s); this intrinsic protein kinase was not present in the contraluminal membrane.

These findings provide direct evidence that the initiating steps in the action of antidiuretic hormone on the kidney take place at the contraluminal pole of the hormone-sensitive target cell and that the late or terminal steps occur at the luminal pole, where they involve an alteration in the level of membrane phosphorylation.

Hormone action at the cellular level involves a sequence of events starting with the selective binding of hormone to receptor and culminating in a biochemical or biophysical effector process (1–3). It is now generally accepted that the activation of adenylate cyclases and protein kinases constitute intermediate steps in the action of several peptide hormones, and it has been hypothesized recently that phosphorylation of plasma membrane components, catalyzed by cyclic 3':5'-adenosine monophosphate (cAMP)-dependent protein kinases, may be implicated in the antidiuretic action of neurohypophyseal hormones (3–8).

Recently we have shown that parathyroid hormone (PT hormone)-sensitive adenylate cyclase is localized in the contraluminal (basal-lateral) but not in the luminal (apical) plasma membranes of rat renal cortical epithelial cells, whereas cAMP-dependent phosphorylation occurs in the luminal but not the contraluminal membranes (9, 10). To determine whether a similar enzymatic distribution occurs in other segments of the nephron, we have investigated the localization of antidiuretic hormone (AD hormone)-sensitive adenylate cyclase and of cAMP-dependent protein kinase in the papillary collecting duct epithelium. The results of these studies indicate that AD hormone-stimulated cAMP generation is localized in the contraluminal plasma membrane, whereas cAMP-mediated membrane phosphorylation is localized in the luminal plasma membrane. The latter localization—in conjunction with the well-established luminal site of AD hormone-induced permeability changes (11–13)—provides evidence in support of the concept that an alteration in the level of phosphorylation of the luminal plasma membrane constitutes an intermediate or terminal biochemical step in the action of AD hormone on the mammalian kidney.

METHODS AND MATERIALS

Myokinase was purchased from Boehringer Mannheim and L-epinephrine was obtained from Serva Biochemicals. Calcitonin (human, synthetic) was a gift from Ciba, and PT hormone (bovine) was the gift of Dr. H. Rasmussen. All neurohypophyseal hormones and analogs were obtained from Dr. R. Walter. All radioisotopes were purchased from New England Nuclear Corp.

Preparation of Membrane Fractions. Freshly excised bovine kidneys were placed in ice-cold ST-buffer (250 mM sucrose, 10 mM triethanolamine-HCl, pH 7.6); all subsequent procedures were carried out at 0–4°. The kidneys were freed from adherent connective tissues and the papillae were removed by careful dissection of the renal pelvis. Six grams of tissue were minced in a small volume of ST-buffer and then diluted to a final volume of 30 ml. Aliquots (10 ml) of the suspension were homogenized in a Dounce homogenizer by five strokes with a loose-fitting pestle. The homogenates were combined and filtered through two layers of cheese cloth and further homogenized by 15 strokes with a tight-fitting pestle. These homogenates were centrifuged at 700 × g for 10 min; the supernatant was centrifuged at 10,000 × g for 10 min. The second supernatant was saved and the pellet resuspended in 5 ml of ST-buffer. Then 10 ml of ST-buffer was added and the suspension centrifuged for 10 min at 10,000 × g. The resulting supernatant was combined with the “sieved” supernatant and the solution was centrifuged at 100,000 × g for 1 hr. The pellet was suspended in 4 ml of electrophoresis buffer (8.5 mM acetic acid, 8.5 mM triethanolamine, and 280 mM sucrose adjusted to pH 7.4 with 2 M NaOH), homogenized with 10 strokes in the tight-fitting Dounce homogenizer, and diluted to a final volume of 10 ml with the electrophoresis buffer. The resulting preparation, observed by electron microscopy to consist of membrane fragments and vesicles, was used to study the properties of papillary plasma membranes prior to electrophoretic separation.

To prepare for electrophoresis, we centrifuged the papillary plasma membranes three times for 10 min at 3000 × g in order to remove aggregates. The supernatant of the last centrifugation was rehomogenized by five strokes in the tight-fitting Dounce homogenizer and subjected to free flow electrophoresis as developed by Hannig (14) and adapted to

Abbreviations: PT hormone, parathyroid hormone; AD hormone, antidiuretic hormone; Ca-ATPase, calcium-stimulated adenosine-triphosphatase; HCO3-ATPase, bicarbonate-stimulated adenosine-triphosphatase.
kidney membranes by Heidrich et al. (15). The membrane fractions obtained on electrophoresis were classified as fraction I (laminal) and fraction II (contraluminal) according to the specific activities of bicharbate-stimulated adenosinetriphosphatase (HCO₃-ATPase) and calcium-stimulated adenosinetriphosphatase (Ca-ATPase), respectively. After dilution with 0.1 M Tris·HCl buffer, pH 7.4, the membranes of separate collection tubes or of combined fractions were obtained by centrifugation for 1 h at 100,000 × g, suspended in 0.1 M Tris buffer and used immediately for determination of enzyme activities. Protein was determined after precipitation with 10% trichloroacetic acid by the method of Lowry et al. (16) using bovine-serum albumin as a standard.

**Adenylate Cyclase Assay.** Adenylate cyclase activity was measured by the method described by Bär and Hechter (17) with the addition of 12.5 μg/ml of myokinase. [³²P]cAMP was separated by chromatography on cellulose (CEL) 300 polyethyleneimine impregnated thin layer plates in 0.3 M LiCl. Radioactivity was measured in a Packard liquid scintillation spectrometer. All assays were performed in triplicate.

**Phosphodiesterase Assay.** Cyclic nucleotide phosphodiesterase activity was determined by measuring the appearance of [³²P]AMP and [³²P]adenosine from [³²P]cAMP. The incubation medium consisted of 0.5 mM [³²P]cAMP (0.5 μCi), 40 mM Tris·HCl buffer, pH 7.5, 4 mM MgCl₂, 0.1 mM ethylenediaminetetraacetate (EDTA), and 30-60 μg of membrane protein in a final volume of 50 μl. The incubation and chromatographic procedures were the same as those described for the adenylate cyclase assay.

**Preparation of Cytosolic Protein Kinase.** Cytosolic, cAMP-dependent protein kinase was prepared from bovine renal papillae following the method described by Kuo et al. (18). The purification procedure was carried through the (NH₄)₂SO₄ precipitation and the dialyzed enzyme solution was rapidly frozen with 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 assay described below.

**Protein Kinase Assay.** Intrinsic protein kinase activity was determined by a modification of the procedure of Kuo et al. (18). The assay mixture contained 50 mM sodium acetate, pH 6.5, 25 μM [γ-³²P]ATP (0.3 μCi), 10 mM MgCl₂, 0.3 mM ethyleneglycol bis[β-aminoethyl]ether]-N,N',N'-tetraacetate (EGTA), 20 mM NaF, 2 mM theophylline, and 50-80 μg of membrane protein in a final volume of 0.2 ml. cAMP was added in concentrations ranging from 0.01 to 5 μM.

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

For measurement of additional phosphorylation of the membrane occurring in the presence of cytosolic protein kinase, 70 μg of the cytosolic enzyme preparation was added to the incubation mixture.

**Determination of [³¹C]ATP Binding.** The amount of ATP that binds to the isolated membrane fractions under the conditions used for phosphorylation was measured by substituting [³¹C]ATP (0.1 μCi) for [γ-³²P]ATP in the standard assay mixture for protein kinase activity.

**Bicarbonate-Activated ATPase.** HCO₃-ATPase was determined in 75 mM Tris·HCl buffer, pH 7.6, containing 3 mM MgCl₂, 3 mM NaATP, and 2 mM ouabain with or without 25 mM Na₂SO₄. Following a 15-min incubation at 37° the samples were boiled for 1.5 min, chilled, and centrifuged. The amount of Pi liberated was determined by the methods of Bartlett (19) and Fiske and Subbarow (20). After correction for the substrate blank, the result of the assay in the absence of sulfite was taken as magnesium-stimulated ATPase activity and the difference between the values with and without sulfite was taken as the HCO₃-ATPase activity. Sulfite is known to stimulate HCO₃-ATPase (21) and we observed that the degree of activation of this enzyme in renal papillary tissue is greater with sulfite than with an equivalent concentration of HCO₃⁻.

**Calcium-Activated ATPase.** Ca-ATPase activity was determined in 75 mM Tris·HCl buffer, pH 7.6, 5 mM CaCl₂, 2 mM ouabain, and 5 mM Tris-ATP in a volume of 500 μl. The samples were incubated for 30 min at 37°; the reaction was terminated by the addition of 500 μl of 10% trichloroacetic acid. After centrifugation, the amount of Pi liberated was determined in aliquots of the supernatant as

**Table 1. Specific activity of marker enzymes during membrane purification* **

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Ca-ATPase</th>
<th>HCO₃-ATPase</th>
<th>Succinate dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>2.4 ± 0.5</td>
<td>1.1 ± 0.2</td>
<td>0.09</td>
</tr>
<tr>
<td>Unfractionated plasma membranes</td>
<td>5.2 ± 0.8</td>
<td>4.6 ± 0.5</td>
<td>0.11</td>
</tr>
<tr>
<td>Fraction I (luminal membranes)</td>
<td>4.5 ± 0.6</td>
<td>11.0 ± 1.4</td>
<td>0.17</td>
</tr>
<tr>
<td>Fraction II (contraluminal membranes)</td>
<td>15.2 ± 3.8</td>
<td>4.1 ± 0.7</td>
<td>0.13</td>
</tr>
</tbody>
</table>

*Data are expressed in μmoles/hr per mg of protein and represent the mean ± standard error of five determinations.
Fig. 2. Effect of arginine vasopressin (AVP) on the adenylate cyclase activity of contraluminal membranes (-- --) and luminal membranes (. . .).

The values were corrected for the ATPase activity found in the absence of any added divalent cation.

Sucinate Dehydrogenase. Succinate dehydrogenase activity was measured according to Gibbs and Reimer (22) after pretreatment of the samples with 0.1% deoxycholate.

RESULTS

Enzyme Activities of Unfractionated Plasma Membranes.

The following enzymes were found in the papillary plasma membrane: Ca-ATPase, HCO₃-ATPase, adenylate cyclase, protein kinase, and phosphodiesterase. The specific activities of Ca-ATPase and HCO₃-ATPase are given in Table 1.

Adenylate cyclase activity was measured under basal conditions and in the presence of maximally stimulating concentrations of arginine vasopressin (AVP); cAMP formation was found to be 18.6 and 39.1 pmol per min/mg of protein, respectively, and increased linearly with time over a 20-min period and with the amount of membrane protein over the range of 40–200 μg. The enzyme was specifically stimulated by neurohypophyseal hormones; calcitonin, epinephrine, and PT hormone were without effect on this preparation. At a concentration of 1 μM the relative stimulation produced by AVP, lysine vasopressin (LVP), arginine vasotocin (AVT), and oxytocin (OXY) was as follows: AVP > LVP > AVT > OXY. The inhibitory analog, arginine vasopressino acid (23), reduced adenylate cyclase activity below basal levels. NaF at a concentration of 10 mM increased enzyme activity by approximately 200%.

A low level of phosphodiesterase activity was found in the papillary plasma membranes. Approximately 0.25 nmol of cAMP was found to be degraded per min/mg of membrane protein when the assay system was charged initially with 25 nmol of cAMP. This level of phosphodiesterase activity did not interfere with the adenylate cyclase assay.

In addition, the plasma membranes were found to contain an intrinsic cAMP-dependent protein kinase that catalyzes the phosphorylation of membrane protein(s). The amount of [³²P]phosphate incorporated into the membranes was 36.4

Fig. 3. Effect of NaF on the adenylate cyclase activity of contraluminal membranes (-- --) and luminal membranes (. . .).

pmol per min/mg of protein under basal conditions. Maximum protein kinase activity attainable in the presence of 1 μM cAMP was approximately twice the basal activity. The time course of phosphorylation was nearly linear up to 5 min and the amount of [³²P]phosphate incorporated in the presence and absence of cAMP was directly proportional to the concentration of membrane protein over a range of 25–120 μg.

When cytosolic protein kinase, partially purified from bovine papillary tissue, was added to the incubation mixture, the amount of [³²P]phosphate incorporated into the membrane in the presence of cAMP was only 13% more than that obtained with the intrinsic enzyme alone.

The possibility was considered that [³²P]phosphate incorporation into the membranes might be overestimated as a result of binding of the entire [³²P]ATP molecule. This possibility was ruled out by experiments in which it was shown that no [¹⁴C]ATP was bound to the membranes either in the presence or absence of cAMP.

Dephosphorylation of papillary plasma membranes was not observed in the presence or in the absence of cAMP during a 10-min interval following a 10-fold dilution of the maximally phosphorylated preparation (ATP concentration reduced from 25 μM to 2.5 μM to limit any further [³²P] incorporation).

Fractionation of Plasma Membranes into Luminal and Contraluminal Components. On free flow electrophoresis, membrane fragments containing AD hormone-sensitive adenylate cyclase and Ca-ATPase (fraction I) were separated from fragments containing cAMP-dependent protein kinase and HCO₃-ATPase (fraction II) (Fig. 1). In analogy to the proximal tubule, Ca-ATPase (24) and HCO₃-ATPase (25) were used as marker enzymes for the contraluminal and luminal plasma membranes, respectively. As shown in Table 1, the enrichment of Ca-ATPase activity in fraction I and the enrichment of HCO₃-ATPase activity in fraction II identified these membrane fractions as contraluminal and luminal, respectively. Inasmuch as HCO₃-ATPase is a constituent of mitochondria as well as the luminal plasma membrane, the succinate dehydrogenase activity of the separated membranes was measured. The low succinate dehydrogenase activity in either of the membrane fractions (Table 1) indicates an absence of significant mitochondrial contamination.

Fig. 2 shows that basal as well as arginine-vasopressin-
stimulated adenylate cyclase activity is localized almost exclusively in the contraluminal membrane fraction where the concentration of arginine vasopressin required for half-maximal stimulation of the enzyme proved to be 40 nM. Similarly the fluoride-stimulated adenylate cyclase activity in the contraluminal membrane fraction was strikingly greater than in the luminal fraction (Fig. 3).

In sharp contrast to the contraluminal localization of adenylate cyclase, the protein kinase activity of the collecting duct epithelial cells is confined to the luminal membrane fraction (Fig. 4). It should be noted (a) that the luminal plasma membrane is itself an intrinsic substrate for its protein kinase and (b) that the addition of cytosolic protein kinase, extracted from the papillary tissue, does not substantially increase the cAMP-dependent phosphorylation of the luminal membrane (i.e., the luminal membrane is a poor substrate for cytosolic kinase) (Fig. 5).

**DISCUSSION**

Recently several groups have isolated and characterized plasma membranes from renal medullary tissue (6, 26-30). In the present study, purified plasma membranes from cells of the papillary collecting duct have been resolved into luminal and contraluminal membrane fractions. We have relied on enzymatic markers to characterize these fractions because, although electron micrographs showed membrane fragments and vesicles in all sections, no clear-cut differences in these elements could be observed when glutaraldehyde-fixed thin sections or negatively stained preparations of the luminal and contraluminal fractions were compared.

Ca-ATPase was selected as the marker for the contraluminal fraction of the papillary plasma membranes because this enzyme was found to be exclusively localized in the basal infoldings of the proximal tubule cells in the rat kidney (24) and because histochemical evidence suggested that Ca-ATPase is present only on the basal and lateral surfaces of epithelial cells of the collecting duct (31). The localization of adenylate cyclase in the membrane fraction containing Ca-ATPase activity reinforces the conclusion that this membrane component was derived from the contraluminal pole, since it is well known that epithelial cells containing adenylate cyclase systems are activated only when the hormone reaches their serosal surface (32-34). In addition, since PT hormone-sensitive adenylate cyclase has been found to be localized in the contraluminal membrane of the proximal tubule epithelial cell (9, 10), it would appear that hormone-sensitive cyclases are similarly localized throughout the nephron.

HCO₃-ATPase was selected as a marker for the luminal membranes of the collecting duct epithelial cell because this enzyme has been shown to be contained exclusively in the brush border fraction of the proximal tubular epithelial cell membrane (25), where it is probably implicated in the process by which proximal tubular urine is acidified; it is reasonable to assume a similar luminal localization in the collecting duct, which is also a site of urinary acidification (35).

Although the classical markers for the luminal and contraluminal membranes in the proximal tubular epithelial cells are alkaline phosphatase and sodium-potassium-stimulated ATPase, respectively, the activity of these enzymes in the papillary plasma membranes was too low to allow either to be used as a marker enzyme.

The failure of AD hormone to elicit its characteristic hydroosmotic action when presented to the luminal surface of the toad bladder (11-13, 34) and renal collecting duct (32) led to the suggestion that the AD hormone receptors are present only at the contraluminal cell surface; however, Havran et al. (36) have shown that a maximal hydroosmotic response can be elicited when AD hormone is presented at the luminal surface of the toad bladder, provided that its concentration in the luminal medium is increased by 4 orders of magnitude. Therefore the question arose whether the hormone at high concentration was penetrating the tight junction or the cell itself to initiate the hormonal reaction sequence from the serosal (contraluminal) cell surface, or whether the hormone was reacting with a low-affinity cyclase system actually present in the luminal membrane. The latter alternative appears now to be ruled out by the finding of a specific localization of adenylate cyclase in the contraluminal membrane of an AD hormone-sensitive cell (Figs. 1 and 2).

Although the response to arginine vasopressin was less than had been observed in other renal medullary preparations

**Fig. 4.** Effect of cAMP on intrinsic protein kinase activity of luminal membranes (-----) and contraluminal membranes (- - -).

**Fig. 5.** Effect of cAMP on the phosphorylation of luminal membranes (-----) and contraluminal membranes (- - -) in the presence of papillary cytosolic protein kinase.
(23, 26–30), the adenylyl cyclase in the papillary membranes was stimulated only by neurohypophyseal hormones, confirming that the AD hormone receptor is highly discriminatory. The decrease in hormone sensitivity may be related to the very extensive homogenization procedure employed in order to obtain a preparation suitable for the electrophoretic separation.

The polarity of the collecting duct epithelial cell is further emphasized by the localization of a cAMP-dependent protein kinase in the luminal membrane. The properties of this enzyme are very similar to those of other cAMP-dependent protein kinases, although there are some important differences. The concentration of cAMP needed for half-maximal stimulation of the intrinsic protein kinase was 0.3 μM, a value higher than that reported for most other protein kinases. However, it should be noted that a cAMP concentration of 0.5 μM was needed to achieve half-maximal stimulation of the papillary cytosolic protein kinase with histone as substrate.

Dousa et al. (6) proposed that the AD hormone-sensitive diffusion barrier of the luminal membrane of collecting duct epithelial cells is a specific substrate for a cytosolic protein kinase. Our results demonstrate that the luminal membrane is indeed preferentially phosphorylated. However, this phosphorylation was found to be catalyzed by the membrane-bound enzyme and was not significantly increased by the presence of the cytosolic protein kinase, suggesting that enzyme–substrate proximity—as well as enzyme-substrate specificity—may be a critical feature of the cAMP-mediated alteration in permeability of the luminal membrane.

That the increased luminal membrane permeability results from a cAMP-mediated increase in the level of phosphorylation is suggested not only by the existence of an intrinsic protein kinase, but also by the absence of cAMP-dependent phosphoprotein phosphatase activity. The latter finding in renal papillary plasma membranes differs from the observation of DeLorenzo et al. (37, 38), who noted activation by cAMP of an intrinsic phosphoprotein phosphatase acting on a specific protein in a membrane fraction from toad bladder. That such a phenomenon also occurs in renal membranes cannot be excluded at the present time because it is possible that dephosphorylation of some specific phosphoprotein(s) may be masked by a predominating concurrent phosphorylation of other membrane proteins.

Considering the enzyme localizations reported above, we are suggesting the following hypothesis for the mechanism of action of antidiuretic hormone in the renal collecting duct: (1) hormone–receptor interaction and activation of adenylyl cyclase at the contraluminal (basal-lateral) cell membrane; (2) generation of cAMP and its translocation to the luminal (apical) cell membrane; (3) activation of luminal membrane-bound protein kinase; (4) alteration of the level of phosphorylation of one or more components of the luminal membrane, leading to an increase in its permeability.

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