Ethacrynic Acid-Sensitive and ATP-Dependent Cl⁻ Transport in the Rat Kidney

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ABSTRACT — Ouabain- and furosemide-insensitive and ATP-dependent Cl⁻ uptake was demonstrated in rat renal membrane vesicles. Such a Cl⁻ uptake activity was prominent in cortical plasma membrane fractions with high activities of Cl⁻-ATPase and Na⁺,K⁺-ATPase. The membrane vesicles accumulated Cl⁻ in an osmotically reactive manner with the following sequence of nucleotide specificity: ATP > ITP > UTP > GTP > CTP. β,γ-Methylene ATP, ADP and AMP had no effect. ATP-dependent Cl⁻ uptake was markedly inhibited by a Cl⁻-ATPase inhibitor, ethacrynic acid (0.3 mM), but not affected by an H⁺-ATPase inhibitor, N,N'-dicyclohexylcarbo- dimide (0.1 mM). These findings suggest that an ethacrynic acid-sensitive and ATP-driven Cl⁻ pump is present in the rat renal cortex.

Although the movement of Cl⁻ in renal tubules has been assumed to be predominantly passive, some active components now appear to participate in tubular reabsorption and secretion of this ion (1, 2). Energy-requiring processes, however, are reportedly related indirectly to Cl⁻ transport systems because they are required for the formation of Na⁺ and K⁺ gradients (2), and at present, there is no evidence for a renal Cl⁻ pump directly linked to the catabolism of ATP.

We recently reported an ATP-driven Cl⁻ pump in rat brain plasma membrane vesicles with ethacrynic acid-sensitive Cl⁻-ATPase activities (3−5). Since Cl⁻-ATPase activity was also found to be considerably higher in the kidney than in tissues other than the brain, we examined the regional and subcellular distribution as well as some characteristics of this renal ATP-dependent Cl⁻ pump.

MATERIALS AND METHODS

Membrane preparations

Microsomal membrane vesicles were prepared from the kidneys of 6−7 week-old male Wistar rats (Kitayama Rabesu). Before the removal of kidneys, the animals were anesthetized with ether and perfused with saline for 10 min. Whole kidneys or separated tissues (cortex, outer medulla and inner medulla) were homogenized in a Teflon-glass homogenizer in an ice-cold buffer solution containing 0.25 M sucrose, 12.5 mM Tris/MES (pH 7.4), 1 mM EDTA·Na₂ and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged (10 min, 1,000 × g; 20 min, 10,000 × g; 30 min, 100,000 × g). The final pellets were suspended in the homogenizing buffer, stored at −80°C and used as microsomal membrane vesicles. Further fractionation was done by sucrose density gradient centrifugation (60
min, 100,000 × g; samples on 0.6/0.8/1.0/1.2 M sucrose). The membrane vesicles banding at each interface or the bottom were collected and diluted 2–20 fold in the homogenizing buffer and then centrifuged (30 min, 100,000 × g). The resulting precipitates were resuspended in the homogenizing buffer and stored at −80°C until they were used. All procedures were done at 0–4°C. The protein concentration was determined by the method of Lowry et al. (6).

Assay of enzyme activities
ATPase activities were determined by the spectrophotometric measurement of the inorganic phosphate liberated as described previously (7, 8). The reaction was carried out for 30 min at 37°C in 200 μl of medium containing 100 mM Tris/MES (pH 7.4), 1 mM EDTA•Na2, 6 mM magnesium acetate, 100 mM NaCl, 10 mM KCl, 6 mM ATP•2Na, 20–30 μg of membrane protein with or without 1 mM ouabain and/or 0.3 mM ethacrynic acid. Following 10-min preincubation, the reaction was started by the addition of ATP and stopped by the addition of 200 μl of ice-cold 10% trichloroacetic acid. Na+,K+-ATPase activity was calculated by subtracting the activity in the presence of 1 mM ouabain from the total ATPase activity. The activity in the presence of 1 mM ouabain was regarded as the Mg2+-ATPase activity. The Mg2+-ATPase activity in the presence of 0.3 mM ethacrynic acid was designated as the anion-insensitive Mg2+-ATPase activity. Cl−-ATPase activity was calculated as the difference between the activities in the presence and absence of 0.3 mM ethacrynic acid.

Glucose 6-phosphatase activity was assayed according to the method of Nordile and Arion (9, 10).

Assay of Cl− uptake
Cl− uptake was assayed as described previously (3–5). The membrane vesicles (0.4 mg of protein) were preincubated at 37°C for 4 min in 200 μl of a mixture which usually contained 50 mM Tris/MES (pH 7.4), 1 mM EDTA, 1 mM ouabain, 2 mM NaN3, 0.1 mM furosemide and 6 mM [36Cl]Cl-Tris (0.1 μCi). The reaction was started by the addition of 3 mM (each) of ATP/Tris (pH 7.4) and magnesium acetate (3 mM ATP/magnesium). Four minutes later, the samples were filtered by a glass microfiber filter (Whatman GF/B) and washed with 15 ml of 100 mM sodium gluconate (pH 7.4). The radioactivity retained on the filters was measured with Scintisol EX-H (Dojin) in a liquid scintillation counter (Packard 2000 CA Tri-Carb).

Assay of H+ accumulation
H+ accumulation in membrane vesicles was assayed by measuring the quenching of the acridine orange fluorescence at 25°C according to the method of Lee and Forte (11). The standard assay medium (2 ml) contained 137 mM KCl, 10 mM Tris/MES (pH 7.4), 2 mM NaN3, 3 mM MgSO4, 1 mM ouabain, 2.5 μM acridine orange and 0.2–0.3 mg protein of membrane vesicles. Following 5-min preincubation, the reaction was started by the addition of 3 mM ATP•2Na; and at the end of the reaction, a protonophore, carbonyl-cyanide-m-chlorophenylhydrazone (CCCP, 20 μM), was added to determine the fluorescence level without an H+ gradient across the vesicle membranes. Fluorescence was measured using a Shimadzu RF-540 spectrofluorometer with an excitation wavelength of 493 nm and an emission wavelength of 530 nm.

Materials
[36Cl]HCl (15.07–15.58 mCi/g) was obtained from Du Pont-New England Nuclear. ATP/Tris, β,γ-methylene ATP, ADP, AMP, ouabain, picrotoxin, CCCP and N,N'-dicyclohexylcarbodiimide (DCCD) were purchased from Sigma; ATP-2Na, ITP-2Na, UTP-3Na, GTP-2Na and CTP-2Na, from Boehringer Mannheim; and phenylmethylsulfonyl fluoride and acridine orange, from Wako. Ethacrynic acid was a gift from Merck, Sharp & Dohme Research Laboratories, and furosemide from Hoechst Japan.
RESULTS

Among the rat tissues tested for microsomal Cl\textsuperscript{-}-ATPase activities, kidney tissues showed high activities comparable with those in the brain (Table 1). Since Cl\textsuperscript{-}-ATPase in the brain is supposed to transport Cl\textsuperscript{-} in an ATP-dependent manner through a process not related to Na\textsuperscript{+},K\textsuperscript{+}-ATPase or Na\textsuperscript{+}/K\textsuperscript{+}/Cl\textsuperscript{-} cotransport (4), the activities of Cl\textsuperscript{-}-ATPase and ATP-dependent Cl\textsuperscript{-} uptake in cortical and medullary microsomes were assayed in the presence of Na\textsuperscript{+},K\textsuperscript{+}-ATPase and/or Na\textsuperscript{+}/K\textsuperscript{+}/Cl\textsuperscript{-} cotransport inhibitors, 1 mM ouabain and/or 0.1 mM furosemide (see legend of Table 2). The activity of Na\textsuperscript{+},K\textsuperscript{+}-ATPase, known to be a plasma membrane-marker enzyme with a characteristic regional distribution in the kidney (12, 13), showed a distribution profile similar to that reported: outer medulla > cortex > inner medulla. The activities of both Cl\textsuperscript{-}-ATPase and ATP-dependent Cl\textsuperscript{-}

### Table 1. Cl\textsuperscript{-}-ATPase activities in the rat tissues

| Tissue        | Cl\textsuperscript{-}-ATPase activity (μmol Pi/mg protein/hr) |
|--------------|-------------------------------------------------------------|
| Kidney       | 3.05 ± 0.39                                                  |
| Heart        | 1.70 ± 0.24                                                  |
| Liver        | 1.14 ± 0.23                                                  |
| Small intestine | 0.52 ± 0.22                                                   |
| Gastric mucosa| 0.11 ± 0.07                                                  |
| Brain-Cerebrum| 4.82 ± 0.17                                                  |
| Cerebellum   | 4.18 ± 0.20                                                  |
| Brain stem   | 3.48 ± 0.75                                                  |
| Spinal cord  | 2.66 ± 0.28                                                  |

Values are means ± S.E. (n = 4–9). Cl\textsuperscript{-}-ATPase activity was assayed as 0.3 mM ethacryninc acid-sensitive Mg\textsuperscript{2+}-ATPase activity in the presence of 1 mM ouabain as described in Materials and Methods.

### Table 2. Activities of Na\textsuperscript{+},K\textsuperscript{+}-ATPase, Cl\textsuperscript{-}-ATPase and ATP-dependent Cl\textsuperscript{-} uptake in the rat kidney

| Region          | Activity (μmol Pi/mg protein/hr) | ATP-dependent Cl\textsuperscript{-} uptake (nmol/mg protein) |
|-----------------|----------------------------------|-------------------------------------------------------------|
|                 | Na\textsuperscript{+},K\textsuperscript{+}-ATPase | Cl\textsuperscript{-}-ATPase                              |
| Cortex          | 6.72 ± 0.30                      | 3.65 ± 0.08                                                 | 2.31 ± 0.48 |
| Outer medulla   | 8.66 ± 0.98                      | 2.28 ± 0.36                                                 | 0.42 ± 0.21 |
| Inner medulla   | 2.94 ± 0.16                      | 1.45 ± 0.17                                                 | NM*          |

Values are means ± S.E. (n = 4–9). The activity of ATP-dependent Cl\textsuperscript{-} uptake was assayed in the presence of 1 mM ouabain and 0.1 mM furosemide. Since 0.1 mM furosemide had no effect on the activity of Cl\textsuperscript{-}-ATPase, it was usually assayed in the presence of 1 mM ouabain. As for the microsomal fraction of rat kidney, the activity of Cl\textsuperscript{-}-ATPase without furosemide was 3.05 ± 0.39 μmol Pi/mg protein/hr and with 0.1 mM furosemide, 3.18 ± 0.06 μmol Pi/mg protein/hr (n = 4). For the other details, see Methods. *NM, not measured because the sample was too small for this assay.
uptake were high in the cortex as compared with those in the medullary regions. Fractionation of cortical microsomes by sucrose density gradient-centrifugation yielded the subfraction with the highest ATP-dependent Cl\(^-\) uptake activity at the 1.0 M/1.2 M interface (Fig. 1). Compared with other subfractions, this fraction also showed higher and lower activities of Na\(^+\),K\(^-\)-ATPase and an endomembrane marker enzyme, glucose-6-phosphatase, respectively.

As shown in Fig. 2, Cl\(^-\) uptake in the presence or absence of ATP was inversely related to medium osmolarity (0.13–0.61 osmol/liter). Extrapolation to infinite medium osmolarity showed that approximately 40% of the Cl\(^-\) accumulated by ATP was bound to vesicular membranes, whereas 60 and 100% of the ATP-dependent and -independent cases, respectively, were taken up into osmotically reactive intravesicular spaces. The present data on ATP-dependent Cl\(^-\) uptake include membrane-bound Cl\(^-\).

Some nucleotides other than ATP also stimulated Cl\(^-\) uptake with a decreasing order of effect: ATP > ITP > UTP > GTP > CTP (Table 3), in which GTP was less effective than in the brain (ATP > GTP > ITP > UTP, Ref. 5). A nonhydrolyzable ATP analogue, β,γ-methylene ATP, and the di- or monophosphate of adenosine did not stimulate Cl\(^-\) uptake.

Ethacrynic acid at a concentration of 0.3 mM markedly inhibited renal ATP-dependent Cl\(^-\) uptake (Table 4), as in the case of ATP-dependent Cl\(^-\) transport in the brain (5), and SITS (0.1 mM), known as an inhibitor for Cl\(^-\)/HCO\(_3\)\(^-\) exchanger (14), partially inhibited Cl\(^-\) uptake. A protonophore, CCCP (10 μM), significantly reduced the Cl\(^-\) uptake, but an
inhibitor of H⁺-ATPase, DCCD (0.1 mM), did not inhibit it (Table 4).

As shown in Fig. 3, the ATP-dependent H⁺-accumulation in membrane vesicles derived from the 1.0/1.2 M sucrose interface fraction was completely inhibited by DCCD (0.1 mM).

Table 3. Effects of nucleotides on Cl⁻ uptake in rat renal microsomes

| Nucleotide (3 mM)     | Cl⁻ uptake (nmol/mg protein) | Total       | Stimulated  | %    |
|-----------------------|------------------------------|-------------|-------------|------|
| None⁴                 | 4.49 ± 0.10                  |             |             |      |
| ATP                   | 6.52 ± 0.10                  | 2.04 ± 0.14 | 100         |
| ITP                   | 6.00 ± 0.56                  | 1.51 ± 0.52 | 74          |
| UTP                   | 5.85 ± 0.31                  | 1.33 ± 0.30 | 65          |
| GTP                   | 5.29 ± 0.19                  | 0.80 ± 0.23 | 39          |
| CTP                   | 5.09 ± 0.24                  | 0.61 ± 0.19 | 30          |
| β,γ-Methylene ATP     | 3.98 ± 0.26                  | −0.47 ± 0.22|          |
| ADP                   | 3.69 ± 0.22                  | −0.80 ± 0.27|            |
| AMP                   | 4.38 ± 0.30                  | −0.11 ± 0.35|            |

Values are means ± S.E. (n = 3 or 4). Cl⁻ uptake was measured as described in the legend of Fig. 1 except that 3 mM each of ATP-2Na, ITP-2Na, UTP-3Na, GTP-2Na, CTP-2Na, β,γ-methylene ATP, ADP or AMP (all adjusted to pH 7.4 with Tris), and 3 mM magnesium acetate were added to start the reactions. *None, 3 mM magnesium acetate without nucleotides was added.
Table 4. Effects of some reagents on Cl− uptake in renal cortical plasma membrane vesicles

| Reagents            | Cl− uptake (nmol/mg protein) |
|---------------------|------------------------------|
|                     | −ATP                         | +ATP                         | △ATP | %    |
| Control (H2O)       | 5.18 ± 0.33                  | 9.65 ± 0.23                  | 4.47 ± 0.26 | 100  |
| 0.3 mM Ethacrynic acid | 5.10 ± 0.27                 | 5.50 ± 0.15                  | 0.40 ± 0.05** | 9    |
| 0.1 mM SITS         | 4.97 ± 0.27                  | 7.43 ± 0.30                  | 2.46 ± 0.18** | 55   |
| Control (5% EtOH)   | 3.89 ± 0.21                  | 6.75 ± 0.31                  | 2.80 ± 0.33  | 100  |
| 0.1 mM DCCD         | 3.39 ± 0.18                  | 5.44 ± 0.27                  | 2.11 ± 0.10  | 75   |
| 10 μM CCCP          | 3.47 ± 0.17                  | 4.24 ± 0.18                  | 0.77 ± 0.11** | 28   |

Values are means ± S.E. (n = 3–8). Cl− uptake was assayed as described in Materials and Methods, using the membrane vesicles from the 1.0/1.2 M sucrose interface fraction. **P < 0.001, as compared with each control using Student’s t-test.

Fig. 3. Effect of DCCD on ATP-dependent H+ accumulation in the 1.0/1.2 M sucrose interface fraction. H+ accumulation was measured by the quenching of acridine orange fluorescence at 25°C, using a protonophore, CCCP (5 μM), to estimate the fluorescence level without an H+ gradient across the vesicle membranes. Upper trace, H+ accumulation in a medium containing 137 mM KCl, 10 mM Tris/MES (pH 7.4), 2 mM NaN3, 3 mM MgSO4, 1 mM ouabain and 0.2 mg of membrane vesicle protein. Following a 5 min preincubation, the reaction was started by the addition of 3 mM ATP-2Na. Lower trace, H+ accumulation in the same medium with 0.1 mM DCCD.

DISCUSSION

In rat kidneys, we observed high activities of Cl−-ATPase and ATP-dependent Cl− uptake comparable with those in the brain (5). Subcellular and regional distributions of both activities as shown in Fig. 1 and Table 2 suggest that ATP-dependent Cl− uptake activity is mainly associated with plasma membranes of cortical nephrons, but scarcely with those of endomembrane origin. Such an ATP-dependent Cl− uptake activity observed in rat kid-
ney can be characterized as follows: 1) renal membrane vesicles transport Cl⁻ into intravesicular spaces via a process of binding to an ATP-requiring Cl⁻ carrier system (Fig. 2), 2) renal ATP-dependent Cl⁻ uptake requires hydrolytic cleavage of the high energy β,γ-phosphate bond of nucleoside triphosphates (Table 3), and 3) neither Cl⁻ /HCO₃⁻ exchanger nor H⁺-ATPase plays a leading role in renal ATP-dependent Cl⁻ uptake (Table 4). Furthermore, CCCP considerably reduced the Cl⁻ uptake, suggesting that H⁺ gradients across the vesicle membranes appeared to act to retain Cl⁻ in the membrane vesicles. The gradients are, however, probably caused by H⁺ movement secondary to the ATP-dependent Cl⁻ uptake, since DCCD at this concentration completely inhibited ATP-dependent primary H⁺ accumulation (Fig. 3) without significant effects on the Cl⁻ uptake (Table 4). All these profiles suggest that an ATP-driven Cl⁻ transport system (Cl⁻ pump) is present in the plasma membranes of cortical renal tubules.

Well-known renal Cl⁻ transport systems such as Na⁺/K⁺/Cl⁻ cotransporters and Cl⁻ /HCO₃⁻ exchangers, mainly exist in the thick ascending limb localized in the outer medulla, and both are inhibited by 0.1 mM furosemide (2). Therefore, even if there were some of these Cl⁻ transporters contaminating the cortical membrane vesicles, they probably did not work under the present experimental conditions (with 0.1 mM furosemide). The renal cortex contains several segments of a nephron: proximal and distal convoluted tubules and the cortical collecting tubule. The latter two segments involve Na⁺ or K⁺ /Cl⁻ cotransport and Cl⁻ /HCO₃⁻ exchanger which are reportedly inhibited by furosemide at concentrations as high as 0.1 mM (2). In the proximal convoluted tubule, the movement of Cl⁻ has been assumed to be predominantly passive and to follow Na⁺ reabsorption (14, 15). However, using luminal microperfusion methods, the participation of an active and cyanide-sensitive Cl⁻ transport in tubular Cl⁻ secretion in this segment has also been demonstrated (16, 17). The ATP-driven Cl⁻ transport demonstrated in this study may provide a biochemical basis for such an active Cl⁻ transport mechanism in the proximal renal tubule.

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