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ATP-driven Ca^{2+} pump in the basolateral membrane of rat kidney cortex catalyzes an electroneutral Ca^{2+}/H^+ antiport

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An ATP-driven Ca^{2+} pump in the basolateral membrane of rat kidney cortex pumps Ca^{2+} out of the cell at the expense of MgATP (K_m = 0.191 mM). This pump has a high affinity for free Ca^{2+} (26 nM). Vanadate, lanthanum, *N*-ethylmaleimide and calmodulin inhibitor R24571 inhibited this pump activity. Dimethyl[2-^{14}C]oxazolidine-2,4-dione ([^{14}C]DMO) was entrapped in the vesicles in association with the ATP-driven Ca^{2+} influx. The ATP-driven Ca^{2+} influx was stimulated by the intravesicular acid pH and an upper convex Lineweaver-Burk reciprocal plot suggested two possible kinetics; one is that this Ca^{2+} pump is an allosteric enzyme with more than 1.72 H^+ binding sites and another is the presence of two Ca^{2+} pumps with different affinities for H^+. Valinomycin study indicated that the ATP-dependent Ca^{2+} transport by the BLMV was electroneutral and voltage independent. These results strongly suggest that the ATP-driven Ca^{2+} pump in the renal basolateral membrane catalyzes an electroneutral Ca^{2+}/H^+ antipo.

Introduction

Over the last decade a considerable amount of knowledge has been accumulating which indicates that the intracellular calcium ion plays a major role in regulating a number of important cellular functions. One of these cellular functions is the regulation of the ionic permeability of the cell membrane. In the renal tubular cell, which is rich in various ion pumps and leaks, cytosolic Ca^{2+} must regulate the transepithelial ion movements. In fact, there are evidences which suggest that an increase in intracellular Ca^{2+} reduces the luminal Na^+ permeability and increases K^+ permeability at the basolateral membrane [1–4]. At the basolateral membrane, there is an Na^+/Ca^{2+} exchanger which transports either ion using the gradient of another ion [5,6]. Thus, intracellular Ca^{2+} regulates the membrane permeability of Na^+ and K^+ changes in these cation transports, in turn, are translated into changes in cytosolic Ca^{2+}.

However, little has been known regarding the role of cytosolic Ca^{2+} on H^+ permeability of cell membrane in the renal tubular cell. In the erythrocyte membrane, studies on reconstituted purified Ca^{2+} pump have demonstrated that the calcium pump catalyzed an electroneutral ATP-

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Abbreviations: EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; BLMV, basolateral membrane vesicles; [^{14}C]DMO, dimethyl[2-^{14}C]oxazolidine-2,4-dione; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethane sulfonic acid; Pipes, 1,4-piperazinediethanesulfonic acid.

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driven Ca^{2+}/H^+ antiport [7–9]. In the kidney, Ca^{2+}-ATPase and Ca^{2+}-pump activities have been studied in the basolateral membrane of the proximal tubular cell from which more than 60% of the filtered Ca^{2+} is reabsorbed [10–12]. Through this side of the membrane, Ca^{2+} has to be pumped out of the cell against an electro-chemical gradient by this Ca^{2+} pump. However, there has been no report regarding the question of whether this Ca^{2+} pump catalyzes the Ca^{2+}/H^+ antiport in this membrane.

In the present study, we provide evidence that the ATP-dependent Ca^{2+} uptake by the inside-out basolateral membrane vesicle (BLMV) takes place electroneutrally and the Ca^{2+}-pump activity has a negative cooperativity with respect to the intravesicular H^+ concentration. The results are consistent with the possibility that the ATP-driven Ca^{2+} pump in the basolateral membrane of rat kidney proximal tubule catalyzes an electroneutral Ca^{2+}/H^+ antiport.

Materials and Methods

Materials. $^{45}CaCl_2$ and [^{14}C]DMO were obtained from Amersham. A23187 was purchased from Calbiochem. ATP-Mg_2, EGTA, Tris, Hepes, Mes, Pipes, Na_3VO_4, $LaCl_3$, and valinomycin were from Sigma. R24571 was purchased from Boehringer Mannheim. Percoll is purchased from Pharmacia. Other chemicals were of highest purity available. All solutions were filtered through 0.45-μm Millipore filters prior to use.

Preparation of basolateral membrane vesicles (BLMV). The BLMV were isolated from the kidney cortex of male Sprague-Dawley rats, each weighing 250–300 g. The kidneys were placed into an ice-cold sucrose buffer (0.25 M sucrose, 0.1 mM phenylmethylsulfonyl fluoride, 5 mM Tris-HCl, pH 7.5) immediately after killing and the cortex was dissected for the isolation of BLMV. The BLMV were isolated by the Percoll gradient procedure, described previously in detail [13]. In experiments in which the intravesicular medium was varied, the BLMV were preloaded by diluting fraction II (the fraction enriched in basolateral membrane by the Percoll gradient [13]) and carrying out the washing procedure at least three times in the described medium. Unless otherwise noted, the only inorganic anion contained in the assay medium is OH^-. The quality of BLMV preparations was evaluated by specific activities and enrichments of marker enzymes such as ouabain-sensitive Na^+/K^+-ATPase for basolateral membrane [14], maltase for brush-border membrane [15], NADH oxidase for endoplasmic reticulum [16], and cytochrome-*c* oxidase for mitochondria [17]. As shown in Table I, the results were essentially the same as reported previously [11] except the Na^+/K^+-ATPase activity which was assayed by a different method in this study. Cortical homogenate and BLMV were treated with 0.1% deoxycholate in order to measure the ouabain-sensitive Na^+/K^+-ATPase activity.

TABLE I

SPECIFIC ACTIVITIES AND ENRICHMENT OF MARKER ENZYMES OF RENAL CORTICAL HOMOGENATES AND BLMV

The specific activities are in μmol/min per mg protein and represent the mean ± S.E. for three or more (n) separate preparations.

	n	Homogenate	BLMV	Enrichment
Na^+/K^+-ATPase	3	0.032±0.001	0.30±0.03	9.29
Maltase	3	0.22 ±0.02	0.30±0.06	1.35
NADH oxidase	4	0.95 ±0.06	1.03±0.10	1.09
Cytochrome-*c* oxidase	4	1.09 ±0.08	0.06±0.01	0.055

BLMV 'sideness'. There are three populations of vesicles in this BLMV preparation by the Percoll gradient method, namely tight right-side out vesicles, tight inside-out vesicles and leaky membranes. Leaky membranes are accessible to ouabain and ATP. The composition of these differently orientated vesicles can be estimated by assessing the inhibitory effect of trypsin on ouabain-sensitive Na^+/K^+-ATPase activity and the effect of detergent on its activity which was proposed by Forbush, III [18]. As a result, an average of 45% of the BLMV preparations were leaky membranes, 38% were tight inside-out vesicles and 17% were tight right-side-out vesicles in the present study. Despite the marked heterogeneity of BLMV in this preparation, only tight inside-out vesicles take Ca^{2+} up in the presence of

extravesicular ATP. This 'sideness' of the ATP binding to the Ca^{2+} pump allowed us to study the kinetics of an ATP-dependent Ca^{2+}-transport using the heterogeneously orientated vesicles.

$^{45}Ca^{2+}$ uptake study. Freshly prepared basolateral membrane vesicles were used. All incubations were carried out at least in triplicate. The uptake of $^{45}Ca^{2+}$ was measured by the Millipore rapid filtration technique [5], using 0.65-μm filters (Millipore DAWP). The filters were presoaked in 'stopping solution', described later, for several hours in order to minimize background radioactivity. To start an ATP-dependent $^{45}Ca^{2+}$ uptake, 10 μl of preloaded vesicles (100 to 150 μg of protein) were added to 290 μl of assay medium containing calcium gluconate, 0.6 μCi of $^{45}CaCl_2$, 0.1 mM EGTA and 50 mM KOH with or without 1.55 mM MgATP in a typical experiment. The calcium gluconate concentration was varied to give the required free Ca^{2+} concentrations at different pH which were calculated by a previously reported computer program [19]. The pH of the assay mixture was maintained at the desired level by the addition of buffers such as Tris, Hepes, Mes and Pipes. Osmolarity was maintained at 300 mosmol/l by the addition of varied concentrations of mannitol. All the assay was performed at 37°C. The uptake was terminated by the addition of 2 ml of an ice-cold 'stopping solution' containing 295 mM mannitol, 2 mM EGTA and 5 mM buffers at the same pH as the assay medium. The membrane vesicles on the filters were rinsed with an additional 6 ml of the cold 'stopping solution'. The radioactive $^{45}Ca^{2+}$ remaining in the vesicles was determined with a liquid scintillation counter. The ATP-dependent $^{45}Ca^{2+}$ uptake was calculated by subtracting the uptake in the absence of ATP but 2 mM $MgCl_2$ from the uptake in the presence of 2 mM ATP-Mg_2 (1.6 mM MgATP). With these conditions of assay, the Ca^{2+} uptake was linear with respect to membrane protein concentration.

[^{14}C]DMO uptake study. In order to study the H^+ transport by the renal BLMV, an uptake of [^{14}C]DMO by the renal BLMV was measured by a free-flow dialysis technique using the micro-dialysis apparatus which was previously described by Kinsella and Aronson [20]. The principle of this technique is as follows. The micro-dialysis apparatus consists of two chambers. The membrane vesicles containing radiolabeled weak acid ([^{14}C]DMO) are placed in the sample chamber, separated by a dialysis membrane from a flowing stream of dialysate in another chamber. The concentration of isotope in the flowing dialysate will be proportional to the concentration of free isotope in the extravesicular medium of the sample chamber. Generation of an extravesicular > intravesicular H^+ gradient ($[H^+]_o > [H^+]_i$), as by the action of a Ca^{2+}/H^+ antiport, will lead to accumulation of the labeled weak acid within the vesicles. The resulting decline in extravesicular concentration of isotope in the sample chamber may then be sensed as a fall in the concentration of isotope in the collected dialysate.

In the present study, 180 μl of membrane suspension (1 to 2 mg protein) in the sample medium were added to the sample chamber with 2.0 μCi (0.20 mM) [^{14}C]DMO. The sample medium contained 235 mM mannitol and 25 mM KOH/15 mM Pipes buffer (pH 6.8). The dialysate was essentially the same as the sample medium. The dialysate flow was maintained at 1.5 ml/min using a peristaltic pump and 0.5 ml fractions were collected and counted by scintillation counter after the addition of 5 ml ACS II (Amersham) to each sample. The reaction was started by the addition of 20 μl 20 mM ATP-Mg_2 (pH was adjusted to 7.4) containing calcium gluconate and 1 mM EGTA in the sample medium. Final free Ca^{2+} concentration was 10 μM and the experiment was performed at 37°C.

Results

Kinetic properties of ATP-dependent Ca^{2+} uptake by the BLMV

Ca^{2+} uptake by the renal BLMV was stimulated by the MgATP in a dose-dependent manner as shown in Fig. 1. An apparent K_m of MgATP was estimated 0.191 mM at the constant 1 μM free Ca^{2+} and the Ca^{2+} uptake became saturated at 1.55 mM MgATP concentration. There was no enhancement of Ca^{2+} uptake by the ATP in the absence of Mg^{2+} (data not shown). However, MgATP-dependent Ca^{2+} uptake was not modified by varying free Mg^{2+} concentration from 10 μM to 5 mM at constant MgATP concentration, indicating that MgATP complex but not free Mg^{2+} is

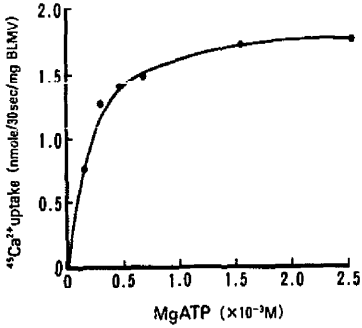

Fig. 1. MgATP dependence of the $^{45}Ca^{2+}$ uptake by the BLMV of rat kidney cortex. The BLMV were loaded with a medium containing 50 mM KOH and 150 mM mannitol. The pH was adjusted to 7.4 by the addition of 50 mM Mes. Free Ca^{2+} concentration was maintained at 1 μM.

the substrate for this Ca^{2+} transport (data not shown). This result is consistent with the finding for the sarcoplasmic reticulum Ca^{2+} pump [21].

The ATP-dependent Ca^{2+} uptake by the BLMV was dependent on the free Ca^{2+} concentration as shown in Fig. 2. An apparent K_m of free Ca^{2+} was estimated 26.2 ± 5.6 nM ($n = 3$) at the constant 1.55 mM MgATP concentration. Since the Ca^{2+} uptake became saturated and steady at 1 μM free Ca^{2+} concentration, every assay was done at

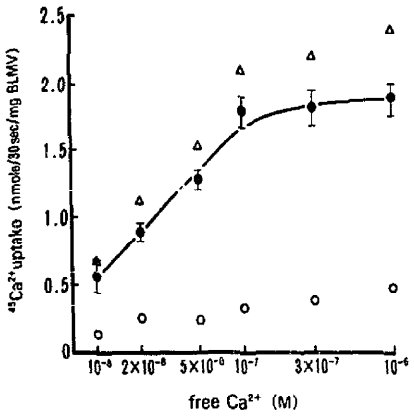

Fig. 2. Ca^{2+} dependence of the ATP-dependent $^{45}Ca^{2+}$ uptake. The $^{45}Ca^{2+}$ uptake was measured at various concentrations of free Ca^{2+} in the absence (○) or presence (△) of 1.55 mM MgATP at pH 7.4. The ATP-dependent $^{45}Ca^{2+}$ uptake is indicated by the closed circle (●); the means ± S.E. ($n = 3$) are presented.

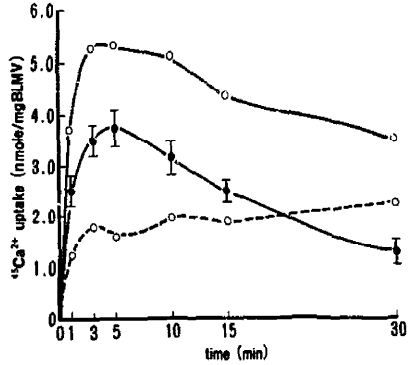

Fig. 3. Time course of $^{45}Ca^{2+}$ uptake by BLMV. The $^{45}Ca^{2+}$ uptake was measured up to 30 min in the absence (○------○) or presence (○———○) of 1.55 mM MgATP. The ATP-dependent $^{45}Ca^{2+}$ uptake is indicated by the closed circle (●———●) ($n = 3$). Free Ca^{2+} was constant at 1 μM.

1 μM Ca^{2+} and 1.55 mM MgATP concentration (37 °C) unless otherwise mentioned.

The time course of Ca^{2+} uptake by the BLMV at 1 μM free Ca^{2+} is shown in Fig. 3. ATP-dependent Ca^{2+} uptake once reached to maximum rapidly at 5 min and decreased gradually, showing 'over-shoot' which is characteristic for an active ion transport.

Effect of calcium ionophore A23187 on the ATP-dependent Ca^{2+} uptake

To test whether the Ca^{2+} uptake by the BLMV represents transport into vesicles rather than membrane binding, the effect of calcium ionophore A23187 on the Ca^{2+} uptake was examined

TABLE II

EFFECT OF A23187 ON $^{45}Ca^{2+}$ UPTAKE BY BLMV

A23187 in an ethanol solution (final 10 μM) was added to the assay mixture 2 min prior to the termination of the $^{45}Ca^{2+}$ uptake. Specific activities are in nmol $^{45}Ca^{2+}$ per mg protein ($n = 3$).

A23187		$^{45}Ca^{2+}$ uptake		
		without ATP	with ATP	ATP-dependent uptake
−	3 min	1.81 ± 0.24	6.24 ± 0.22	4.43 ± 0.46
−	5 min	1.73 ± 0.14	7.73 ± 0.21	6.00 ± 0.28
+	3 min	1.89 ± 0.39	1.43 ± 0.20	none
+	5 min	1.88 ± 0.12	1.79 ± 0.12	none

(Table II). When 10 μM A23187 was added into the assay mixture 2 min prior to the termination of the reaction, the difference in uptake of Ca^{2+} between the assay mixture with and without MgATP was completely dissipated. However, A23187 did not affect the Ca^{2+} uptake in the absence of MgATP. These results indicated that MgATP transported Ca^{2+} into the vesicles and the Ca^{2+} uptake by the BLMV in the absence of MgATP is solely the binding to the membrane.

Effects of various inhibitors on the ATP-dependent Ca^{2+} uptake

Table III shows the various inhibitors on the ATP-dependent Ca^{2+} uptake by the renal BLMV. 50 μM vanadate inhibited 37.1 ± 1.8% of the ATP-dependent Ca^{2+} uptake. NaN_3, an inhibitor of the mitochondrial Ca^{2+}-ATPase, did not affect the Ca^{2+} uptake, indicating this Ca^{2+} pump is not originated from mitochondria. 0.2 mM lanthanum inhibited the Ca^{2+} uptake by 38.5 ± 8.3% of the activity. R24571, an inhibitor of calmodulin, inhibited 48.0 ± 26.9% of the ATP-dependent Ca^{2+} uptake at a concentration of 0.1 mM, suggesting that this Ca^{2+} transport is calmodulin dependent. It is of interest that *N*-ethylmaleimide showed a marked inhibition on this Ca^{2+} pump. *N*-Ethylmaleimide modifies SH group of enzyme and has been shown to inhibit the conversion of ADP-sensitive phosphoenzyme to ADP-insensitive phosphoenzyme in the sarcoplasmic reticulum Ca^{2+} pump [22]. *N*-Ethylmaleimide also inhibits H^+-ATPase activity [23]. These results demonstrated that the Ca^{2+} pump in the renal BLMV shares the common enzyme characteristics of the Ca^{2+} pump in the other organs.

TABLE III

EFFECTS OF VARIOUS INHIBITORS ON THE ATP-DEPENDENT $^{45}Ca^{2+}$ UPTAKE

The BLMV (80–120 μg) were preincubated with each inhibitor except *N*-ethylmaleimide (NEM) and $LaCl_3$ at 37 °C (pH 7.4). The preincubation time was 5 min for Na_3VO_4 and NaN_3 and 10 min for R24571. The specific activities are in nmol $^{45}Ca^{2+}$/60 s per mg protein for study A ($n = 3$) and nmol $^{45}Ca^{2+}$/30 s per mg for study B ($n = 3$).

	$^{45}Ca^{2+}$ uptake		
	without ATP	with ATP	ATP-dependent uptake
Study A.R24571			
0	1.01 ± 0.08	3.47 ± 0.19	2.46 ± 0.12
10^{-5} M	1.13 ± 0.04	3.46 ± 0.47	2.32 ± 0.48
$5 \cdot 10^{-5}$ M	0.79 ± 0.05	2.84 ± 0.15	2.06 ± 0.10
10^{-4} M	0.84 ± 0.06	1.64 ± 0.25	0.80 ± 0.25
Study B.			
Control	0.93 ± 0.12	2.44 ± 0.21	1.51 ± 0.26
Na_3VO_4 5 μM	1.07 ± 0.04	2.43 ± 0.22	1.36 ± 0.23
50 μM	0.88 ± 0.13	1.82 ± 0.05	0.94 ± 0.14
NaN_3 1 mM	1.02 ± 0.04	2.51 ± 0.19	1.50 ± 0.18
NEM 0.5 mM	0.86 ± 0.09	1.13 ± 0.06	0.25 ± 0.06
$LaCl_3$ 0.2 mM	0.79 ± 0.11	1.68 ± 0.10	0.89 ± 0.01

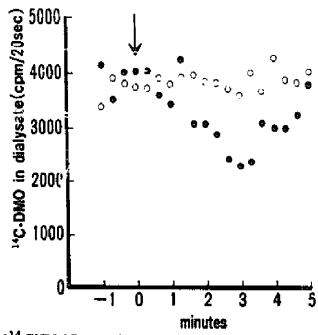

Fig. 4. [^{14}C]DMO uptake by BLMV measured by a free-flow dialysis method. The radioactivity of [^{14}C]DMO was measured in the flowing dialysate every 20 s. 20 μl of 2 mM ATP-Mg_2 (●) or 2 mM ADP-Mg_2 (○) were added to the assay mixture (indicated by the arrow) to initiate the reaction. Free Ca^{2+} was maintained at 10 μM and the assay was performed at pH 6.8 and 37 °C. The decline in radioactivity in the dialysate means uptake of [^{14}C]DMO by BLMV.

[^{14}C]DMO uptake by the BLMV

DMO is a weak acid and can be taken up by the vesicles when the intravesicular space is alkalinized. Fig. 4 is one of the typical experiments. The radioactivity of [^{14}C]DMO started to decrease from the dialysate at 1 min after the addition of MgATP and reached to a minimum at 3 min. The radioactivity returning to the initial level by 5 min. There was no significant change in the radioactivity in the dialysate when MgADP was added to the assay mixture instead of MgATP. When Ca^{2+} was omitted from the assay mixture, there was no decline in the radioactivity even in the presence of

the MgATP (data not shown). This result suggests that intravesicular space was alkalinized accompanying the MgATP-driven Ca^{2+} transport into the vesicles. However, this result cannot exclude the possibility that the DMO was taken up by the vesicles by the inside positive membrane potential which was created by the Ca^{2+} influx.

Effect of the membrane potential on the ATP-dependent Ca^{2+} uptake

The potassium ionophore valinomycin generates a membrane potential in the presence of a potassium gradient across the vesicles. The ATP-dependent Ca^{2+} uptake was not affected by either an outside negative K^+ diffusion potential (50 mM K^+ in the extravesicular medium and 50 mM tetramethylammonium$^+$ in the intravesicular medium) or an inside negative K^+ diffusion potential (50 mM tetramethylammonium$^+$ in the extravesicular medium and 50 mM K^+ in the intravesicular medium) as shown in Table IV. The concentration of valinomycin was decided by the study on Na^+/Ca^{2+} exchange by the BLMV of rat kidney cortex which has been proven to be electrogenic [5]. Fig 5 demonstrated 0.4 μg/ml of valinomycin was enough high to stimulate $^{45}Ca^{2+}$ uptake by the BLMV in the presence of intravesicular > extravesicular Na^+ gradient (Na^+/Ca^{2+} exchange). Since extravesicular medium contained an equimolar K^+ instead of Na^+, valinomycin generated an inside positive membrane potential and stimulate 3 Na^+/1 Ca^{2+} exchange. However,

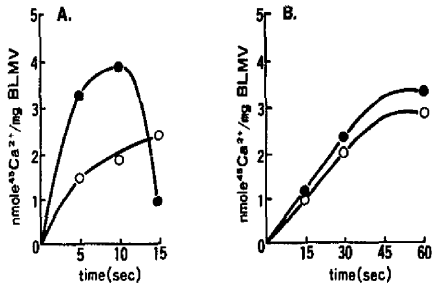

Fig. 5. Effect of valinomycin on Na^+/Ca^{2+} exchange and ATP-dependent Ca^{2+} uptake by the BLMV of rat kidney cortex. Fig. 5A depicts the effect of valinomycin on the $^{45}Ca^{2+}$ uptake by the BLMV when an intravesicular > extravesicular Na^+ gradient was present. The BLMV were loaded with a medium containing 100 mM NaOH and 100 mM Mes (pH 7.4) and were preincubated with the assay mixture containing valinomycin (0.4 μg/ml) in 2.5% ethanol (●) or only 2.5% ethanol as a control (○) for 60 min at 37°C. The $^{45}Ca^{2+}$ uptake was initiated by the addition of 10 μl of this loaded vesicles to the assay mixture containing 100 mM KOH and 100 μM free Ca^{2+} (pH 7.4) [5]. Fig. 5B depicts the ATP-dependent $^{45}Ca^{2+}$ uptake in the absence (○) or presence (●) of valinomycin. The BLMV were loaded with a medium containing 50 mM KOH/50 mM Mes and 150 mM mannitol (pH 7.4). The assay mixture also contained calcium gluconate and 0.1 mM EGTA with or without MgATP. The data represent the mean of three different preparations.

the same concentration of valinomycin was ineffective to stimulate ATP-dependent $^{45}Ca^{2+}$ uptake in the presence of 50 mM K^+ at both side of vesicles (Fig. 5).

It is of interest that replacement of K^+ in the extravesicular medium by tetramethylammonium$^+$ decreased the rate of ATP-dependent Ca^{2+} uptake with or without valinomycin (Table IV). Table V also shows the decreased ATP-dependent Ca^{2+} uptake when the K^+ was replaced with Na^+ or tetramethylammonium$^+$ in the assay medium on both sides of vesicles with or without valinomycin. However, ATP independent $^{45}Ca^{2+}$ uptake was not influenced by the monovalent cations in the medium.

TABLE IV

EFFECT OF THE MEMBRANE POTENTIAL ON THE ATP-DEPENDENT $^{45}Ca^{2+}$ UPTAKE

BLMV (80–120 μg) were preincubated with or without valinomycin (0.4 μg/ml) in ethanol (final 0.9%) for 60 min at 37°C. In order to generate a K^+ gradient across the membrane, K^+ was replaced with equimolar tetramethylammonium$^+$ (TMA^+) in either side of the vesicle. The specific activities are in nmol $^{45}Ca^{2+}$/min per mg protein ($n = 3$).

$[K^+]_{in}$	$[K^+]_{out}$	Valinomycin	ATP-dependent $^{45}Ca^{2+}$ uptake
0 mM	50 mM	–	2.87±0.21
		+	2.84±0.17
50 mM	0 mM	–	1.62±0.18
		+	1.68±0.28

Effect of the pH on the MgATP-dependent Ca^{2+} transport

The effect of pH of the assay medium on the Ca^{2+} uptake by the BLMV is shown in Table VI. ATP-dependent Ca^{2+} transport was stimulated by an intravesicular acid pH but was not affected by

TABLE V

EFFECT OF MONOVALENT CATIONS AND POTASSIUM IONOPHORE VALINOMYCIN ON THE $^{45}Ca^{2+}$ UPTAKE BY BLMV

$^{45}Ca^{2+}$ uptake was measured in the presence of an equimolar K^+, tetramethylammonium$^+$ (TMA^+) or Na^+ with or without valinomycin. The pH of the assay mixture was adjusted by the combination of 50 mM KOH, tetramethylammonium hydroxide or NaOH with Mes or Pipes at pH 7.4.

Cation	Valinomycin	$^{45}Ca^{2+}$ uptake	
		ATP-independent	ATP-dependent
K^+	−	1.16 ± 0.05	1.98 ± 0.17 (A)
	+	1.12 ± 0.19	1.97 ± 0.09
Na^+	−	0.82 ± 0.15	1.13 ± 0.09 (B)
	+	0.98 ± 0.21	0.84 ± 0.11
TMA^+	−	1.20 ± 0.12	0.70 ± 0.11 (C)
	+	1.25 ± 0.13	0.58 ± 0.09

(A) vs. (B) and (A) vs. (C), significant at $P < 0.05$ by Student's t-test for paired data ($n = 4$).

the extravesicular pH. The dependency of ATP-dependent Ca^{2+} transport on the intravesicular H^+ concentration, studied at constant 1 μM free Ca^{2+}, is depicted in Fig. 6. A Lineweaver-Burk reciprocal plot showed an upward convex pattern, suggesting either a negative cooperativity with respect to the intravesicular H^+ concentration or the presence of two Ca^{2+} pumps with different affinities for H^+. In the former case, the apparent n value can be calculated to be 0.56 ± 0.02 ($n = 4$). In the latter case, K_m (H^+) can be calculated to be 4.53 ± 0.82 nM (pH 8.34) for the high-affinity pump and 24.1 ± 5.9 nM (pH 7.62) for the low affinity one.

TABLE VI

EFFECT OF pH ON ATP-DEPENDENT $^{45}Ca^{2+}$ UPTAKE BY BLMV

The intravesicular pH was varied from 6.8 to 8.0 with an extravesicular pH 6.8, 7.4 or 8.0. Free Ca^{2+} concentration was constant at 1 μM at variable extravesicular pH. The pH was adjusted by the addition of Pipes (pH 6.8), Mes (pH 7.4) or Hepes-Tris (pH 8.0) to 50 mM KOH (37 °C). B vs. C and D vs. D, significant at $P < 0.05$ by the student's t test for the paired data ($n = 5$). A vs. B, C vs. E and D vs. F, not significant.

	pH		ATP-dependent $^{45}Ca^{2+}$ uptake (nmol/30 s per mg)
	intravesicular	extravesicular	
A.	6.8	6.8	1.63 ± 0.18
B.	6.8	7.4	1.64 ± 0.09
C.	7.4	7.4	1.28 ± 0.03
D.	8.0	7.4	0.87 ± 0.09
E.	7.4	6.8	1.11 ± 0.10
F.	8.0	8.0	0.85 ± 0.11

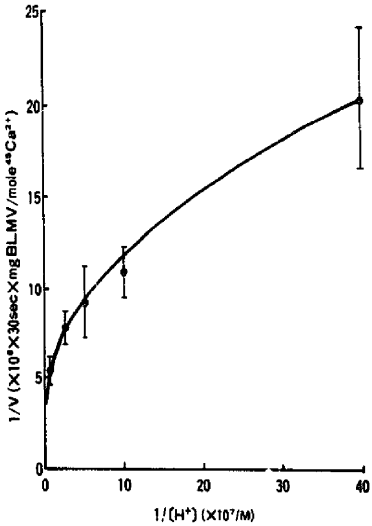

Fig. 6. Lineweaver-Burk reciprocal plot of ATP-dependent $^{45}Ca^{2+}$ uptake against intravesicular H^+ concentration. The plot indicates a negative cooperativity with respect to the intravesicular H^+ concentration ($n = 4$).

Discussion

The main fraction of the basolateral membrane which was purified from the rat kidney cortex in this study is derived from the proximal tubule as previously reported [5]. Ca^{2+} can enter the cell through the brush-border membrane of the proximal tubule by passive diffusion. However, cytosolic Ca^{2+} must be driven out of the cell through the basolateral membrane against the electrochemical gradient. The present study demonstrates the presence of a Ca^{2+} pump which utilizes the energy of ATP for the active transport. Actually, the true substrate of this Ca^{2+} pump is an MgATP complex rather than free ATP. For this reason, this active Ca^{2+} transporter requires Mg^{2+} for the activity but free Mg^{2+} did not stimulate the activ-

ity at all. The calcium ionophore, A23187, completely abolished the ATP-dependent Ca^{2+} uptake by the BLMV but did not affect the Ca^{2+} uptake which was not driven by ATP. This result indicates that the ATP-driven Ca^{2+} uptake is the transport into the intravesicular space but not the binding to the membrane. In the absence of ATP, however, the Ca^{2+} uptake by the BLMV is solely binding to the membrane.

We found only one affinity of the Ca^{2+} pump with respect to the Ca^{2+} binding. The apparent K_m was 26 nM of free Ca^{2+}. This K_m value is at the lower side of the cytosolic Ca^{2+}. The previously reported K_m value of the ATP-dependent Ca^{2+} transport is in the range between 70 nM and 130 nM in rat kidney [10,12] and out value is lower than those values. In the rabbit kidney proximal tubule, Viera et al. [12] reported the presence of high-affinity ($K_m = 1.8$ μM of Ca^{2+}) and low-affinity ($K_m = 250$ μM of Ca^{2+}) ATP-dependent Ca^{2+} transporter. They also reported that free Mg^{2+} stimulated the Ca^{2+}-pump activity in the presence of saturated ATP which is against our finding in this study. We also previously demonstrated the high- and low-affinity Ca^{2+}-dependent ATPase activities in the basolateral membrane of rat kidney cortex [11]. However, we now failed to find the low-affinity Ca^{2+}-pump activity in the same preparation. This is one of the discrepancies between the ATP-dependent Ca^{2+} pump and the Ca^{2+}-dependent ATPase activity in the same preparation of basolateral membrane of rat kidney cortex.

In our previous study [11], Ca^{2+}-dependent ATPase activity was inhibited by free Mg^{2+} and CaATP complex. The enzyme activity was not inhibited by vanadate which is a common inhibitor of the Ca^{2+} pump in various organs [23]. However, the ATP-dependent Ca^{2+} pump requires Mg^{2+} for the activity and is inhibited by vanadate. In addition, both enzyme activities do not share a number of characteristics. Such a large discrepancy forces us to conclude the Mg^{2+}-independent Ca^{2+}-dependent ATPase activity is not the expression of the ATP-dependent Ca^{2+} pump in the basolateral membrane of rat kidney cortex. There is also a report that high-affinity (Ca^{2+} + Mg^{2+})-ATPase activity is not a Ca^{2+}-pumping ATPase in the liver plasma membrane [24].

The goal of this study is to determine whether the ATP-driven Ca^{2+} pump catalyzes an electroneutral Ca^{2+}/H^+ antiport. We measured the uptake of radiolabelled weak acid [^{14}C]DMO by the free flow dialysis method to examine the H^+ efflux out of the vesicle in association with the Ca^{2+} influx. The result showed the transient uptake of this weak acid only in the presence of both Ca^{2+} and MgATP in the assay mixture, suggesting the occurrence of a Ca^{2+}/H^+ exchange. There were two arguments arose from this result which should be overcome. Initially, the [^{14}C]DMO uptake could be caused by the decline in extravesicular pH due to ATP hydrolysis instead of an intravesicular alkalinization. However, if this were indeed the case, decreased radioactivity should have also been observed even in the absence of Ca^{2+} because this membrane contained a Mg^{2+}-ATPase activity. Secondly, the peak of DMO uptake occurred earlier than that of the ATP-driven Ca^{2+} uptake. Although, the reason for this discrepancy could be explained by the leakiness of this membrane to H^+, the possibility should be considered that a DMO also moved into the vesicle because of the inside positive membrane potential.

Since purification of the Ca^{2+} pump has not been successful in the renal tubular cell, isolated membrane vesicles were employed in this study. This membrane contains various anion exchangers which must modify the H^+ movement in this study [25]. This is the reason why any inorganic anion except OH^- was excluded from the assay mixture in most of the experiments. However, the possibility still exists that the H^+ movement could have occurred because of the potential difference generated by the Ca^{2+} movement across the vesicle.

If the ATP-dependent Ca^{2+} transport is electrogenic, H^+ can enter the cell due to an inside negative potential created by the Ca^{2+} efflux out of the cell through the basolateral membrane. This possibility was eliminated by the valinomycin study. In the presence of 50 mM K^+ in both sides of the vesicle, the potassium ionophore valinomycin did not increase the velocity of the ATP-dependent Ca^{2+} uptake. Under the same condition, valinomycin stimulated an Na^+/Ca^{2+} exchange in the same preparation of the BLMV which has been proved to be electrogenic [5]. Neither an

inside negative nor an inside positive potential modified the ATP-dependent Ca^{2+} transport by the BLMV. In the rabbit proximal tubules, Viera et al. [12] reported the inhibition of ATP-dependent Ca^{2+} pump activity by valinomycin. In their study, the concentration of valinomycin was much higher than ours, considering that the effect might not be specific to potassium permeability. 0.4 μg/ml (0.36 μM) of valinomycin has been proved to stimulate an Na^+/Ca^{2+} exchange by rat BLMV [19]. These previous reports have given credit to the effectiveness of valinomycin as a tool for generating a membrane potential in BLMV of rat kidney cortex. These results strongly suggest that the ATP-dependent Ca^{2+} efflux out of the proximal tubular cell is an electroneutral and voltage-independent process and completely agrees with the results of the Ca^{2+} pump in the erythrocyte membrane [7,8].

The present study also revealed that the Ca^{2+}-pump activity was influenced by extravesicular or intracellular monovalent cations. Potassium gave higher activity for the Ca^{2+} pump than Na^+ or TMA^+. One could claim that the decreased pump activity in the presence of Na^+ was caused by an enhanced Ca^{2+} efflux due to the Na^+/Ca^{2+} exchange activity. However, this was not the case in this experiment because an ATP-independent $^{45}Ca^{2+}$ uptake did not decrease by the presence of Na^+ in this assay condition. The 'sidedness' of K^+ effect on the Ca^{2+} pump has been studied in the sarcoplasmic reticulum by Shigekawa et al. [26]. They concluded that the sarcoplasmic reticulum Ca^{2+} pump was stimulated by the cytosolic K^+ indirectly by compensating the charge imbalance caused by the electrogenic Ca^{2+} movement. The present valinomycin study did not agree with their conclusion in the kidney tubular cell.

If the Ca^{2+} pump catalyzes an electroneutral Ca^{2+}/H^+ antiport, an H^+ binding site should face into the extracellular side of the membrane (intravesicular side of the inside-out vesicle). The result demonstrates that the Ca^{2+} uptake by the BLMV is only dependent on the intravesicular pH in the presence of MgATP but is not influenced by the extravesicular pH nor the intravesicular > extravesicular H^+ concentration gradient. This fact suggests the presence of an H^+ binding site at the intravesicular side of the inside-out BLMV. An analysis on an upward convex pattern of Lineweaver-Burk plot suggests two possible mechanisms of H^+ binding. One is the presence of a negative cooperativity with respect to intravesicular H^+ concentration. In this case, this Ca^{2+} pump should be an allosteric enzyme with more than 1.72 H^+ binding sites, which agrees quite well with the stoichiometry of 1 Ca^{2+}/2 H^+ electroneutral antiport. Another possible interpretation is the presence of two Ca^{2+} pumps with different affinities for H^+. However, the K_m value of high-affinity pump was calculated to be pH 8.34 which is far beyond the physiological pH of extracellular space. At present, it is unable to differentiate these two kinetic possibilities.

In summary, the present study demonstrates that ATP-dependent Ca^{2+} uptake by renal BLMV takes place electroneutrally and is stimulated by the intravesicular acid pH. The data suggests that the ATP-dependent Ca^{2+} pump catalyzes an electroneutral Ca^{2+}/H^+ antiport in the basolateral membrane of rat kidney proximal tubules.

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