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Calcium transport in basolateral plasma membranes from kidney cortex of Milan hypertensive rats

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Ca^{2+} transport was investigated in basolateral plasma membranes (BLM) isolated from kidney cortex of the Milan strain of genetically hypertensive rats (MHS) and their normotensive controls (MNS) during a pre-hypertensive stage (age 3–4 weeks). It was found that the V_{max} of ATP-dependent Ca^{2+} transport (in the presence of calmodulin) was about 16% lower in MHS than in control rats. In membranes from MNS rats which had been isolated in the presence of EGTA, the ATP-dependent Ca^{2+} transport showed a hyperbolic Ca^{2+} concentration dependence, a high K_m (Ca^{2+}) and a low V_{max}; upon addition of exogenous calmodulin, the kinetics became sigmoidal, the K_m (Ca^{2+}) was decreased and the V_{max} was increased. In membranes from MHS rats, the Ca^{2+} concentration dependence of ATP-driven Ca^{2+} transport was sigmoidal and the Ca^{2+} affinity was high in the absence of added calmodulin. Addition of exogenous calmodulin to these membranes resulted in an increase in V_{max}, but no change in other kinetic parameters. Low-affinity hyperbolic kinetics of Ca^{2+} transport could only be obtained in MHS rats if the membranes were extracted with hypotonic EDTA and hypertonic KCl. These data suggest that the plasma membrane Ca^{2+}-ATPase, which catalyses the ATP-dependent Ca^{2+} transport, exists in BLM of pre-hypertensive MHS rats predominantly in an activated, high-affinity form.

Introduction

Many working hypotheses have been proposed for explaining the mechanisms of essential hypertension. From the point of view of cellular disorders present in this condition, two of them seemed especially interesting to us. The first one suggests that, in different strains of genetically hypertensive animals, as well as in at least some humans, there is a generalized, genetically transmitted defect in the structure and function of plasma membranes which affects the transport of several ions, including calcium (for a review, see Ref. 1). The second one stresses the well-documented role of renal retention of sodium in the development of hypertension, and suggests that high blood pressure results from a response of volume-regulatory mechanisms to a primary renal defect (for reviews, see Refs. 8, 9).

In the Milan strain of genetically hypertensive rats, the defects of both types are apparent. Hy-

Abbreviations: HEDTA, *N*-hydroxyethylenediaminetetraacetic acid; Mops, 4-morpholinepropanesulfonic acid; BLM, basolateral membranes; MHS and MNS, Milan strain of genetically hypertensive rats and their normotensive controls, respectively.

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pertension in this strain 'goes with the kidney': it can be produced in control rats by transplantation of kidneys from hypertensive rats and ameliorated in hypertensive rats by transplantation of normal kidneys [9]. The development of hypertension is associated with a progressive retention of sodium [10]. An increased permeability of the brush-border membranes to Na^+ in this strain has been reported [11]. On the other hand, several abnormalities in the erythrocytes, including ATP-dependent Ca^{2+} transport, have been described in this strain, which suggests the presence of an intrinsic, genetically transmitted membrane defect [7,12]. Abnormalities of Ca^{2+} binding and transport have also been described in plasma membranes isolated from several tissues of different strains of spontaneously hypertensive rats [2–7].

Na^+ reabsorption in kidney cells is regulated by intracellular Ca^{2+}, which decreases the permeability of the brush-border membranes to Na^+ and reduces the transepithelial Na^+ transport (for a review, see Ref. 13). One of the key systems that regulates the intracellular Ca^{2+} concentration in kidney cells is the calmodulin-dependent Ca^{2+}-transporting ATPase, located in the basolateral plasma membranes (BLM) [14–18]. Therefore, we investigated the Ca^{2+} transport in isolated renal plasma membranes from Milan hypertensive rats, since any alterations in this process might secondarily affect the reabsorption of Na^+ in the kidney. Our results show that the pattern of calmodulin activation of the Ca^{2+}-transporting ATPase is different in normal and in hypertensive animals. In renal plasma membranes of young rats from the hypertensive strain, in contrast to their normotensive controls, the Ca^{2+}-transporting ATPase exists largely in an activated, high-affinity form.

Materials and Methods

Animals. The experiments were carried out on male Milan hypertensive rats and their age- and weight-paired normotensive controls. Most experiments were performed in young animals (aged 20–21 days, body weight 60–80 g) in their prehypertensive stage. The animals had free access to food and water. They were killed by cervical dislocation and the kidneys were rapidly removed and chilled in ice-cold 0.25 M sucrose.

Isolation and storage of renal basolateral membranes (BLM). The membranes were isolated by a slightly modified version of a Percoll centrifugation procedure [17,19]. Slices of outer kidney cortex, about 1 mm thick, were cut with a razor blade. The tissue from 5 rats (about 1.2 g) was homogenized with a loosely fitting glass-Teflon Potter homogenizer in 35 ml of 0.32 M sucrose, 10 mM triethanolamine, 0.1 mM EGTA (pH 7.4) containing 0.05 mM each of the following proteinase inhibitors: phenylmethylsulfonyl fluoride (PMSF), *N*-tosyl-L-lysinechloromethyl ketone (the hydrochloride) (TLCK), tosyl-L-phenylalaninechloromethyl ketone (TPCK), and 1,10-phenantroline. The proteinase inhibitors were prepared as a stock solution of 100 mM each in dimethyl sulfoxide, which was added to the homogenization medium immediately before homogenization. The homogenate was centrifuged for 10 min at $2500 \times g$ and the pellet was discarded and the supernatant was centrifuged for 20 min at $20000 \times g$. The supernatant was discarded and the pink fluffy layer that formed on top of the pellet was taken up in about 30 ml of 0.32 M sucrose/10 mM triethanolamine/0.1 mM EGTA (pH 7.40) (without proteinase inhibitors, which were added during homogenization only). The membranes were homogenized with 10 strokes of a Potter homogenizer at full speed and centrifuged for 20 min at $20000 \times g$. The fluffy layer on top of the pellet was taken up in about 25 ml of 0.25 M sucrose/10 mM triethanolamine/0.1 mM EGTA (pH 7.4) and homogenized, and this was filled up to exactly 30 ml and mixed with 4.1 ml of 90% (v/v) stock Percoll solution in 0.25 M sucrose. The mixture was centrifuged in an SS-34 Sorvall rotor with an integrator set to $8.00 \cdot 10^9\ \Omega^2 r$. For analysis of the gradient, 1.5 ml fractions were collected from the top of the gradient with a Haake-Büchler automatic gradient pump. Routinely, the first 3.5 ml from the top of the gradient were discarded and the next 7.5 ml, which contained the BLM fraction, were collected. The BLM fraction was diluted to 25 ml with 100 mM KCl/50 mM K · Mops (pH 7.4)/5 mM $MgCl_2$, and centrifuged for 50 min at $110000 \times g$. The BLM which sedimented on top of a glassy Percoll pellet were suspended in about 1 ml of a 'storage solution', which contained 100 mM mannitol/100 mM

KCl/20 mM K · Mops (pH 7.0)/3 mM $MgCl_2$/5 mM EGTA/1 mM dithiothreitol/1 mM ATP/15% (w/v) glycerol. The suspension was homogenized with a syringe fitted with a fine needle, divided into 0.2 ml samples and stored in a liquid-nitrogen tank. The protein concentration was 4–6 mg/ml. Before each experiment, the membranes were thawed slowly on ice, diluted to 1.5 ml with 100 mM KCl/50 mM K · Mops (pH 7.4)/5 mM $MgCl_2$/1 mM dithiothreitol, and centrifuged for 15 min in an Eppendorf centrifuge. The washing step was repeated once, and the final membrane pellet was suspended with a syringe in about 0.3 ml of the same solution.

Na^+/K^+-ATPase activity in the BLM suspended in the 'storage solution' did not change appreciably when stored for 7 days at 0 °C. Freezing, thawing and washing resulted in a decrease of the Na^+/K^+-ATPase activity by 15–20% as compared to fresh, unfrozen samples, but the activity did not change further over the several hours required for the experiment. Freezing, thawing and washing resulted in removal of 30–50% of the protein, presumably loosely bound non-membrane proteins. The ATP-dependent Ca^{2+} transport was, as a rule, 2–3-times higher in frozen-thawed than in fresh membrane preparations, probably due to removal of non-membrane protein as well as increased membrane vesiculation during the procedure. The membranes could be stored in a liquid nitrogen tank for at least 3 months without appreciable loss of ATP-dependent Ca^{2+}-transport activity.

Calmodulin depletion. To deplete the membranes of endogenous calmodulin, a 0.2 ml sample of thawed BLM was diluted into 14 ml of 10 mM K · Mops (pH 7.4)/5 mM EDTA/1 mM dithiothreitol, and stirred for 30 min at 0 °C. Then, 14 ml of 1 M KCl was added and stirring was continued for another 30 min. The membranes were collected by centrifugation for 50 min at 110000 × *g*, and suspended in a solution containing 100 mM KCl/50 mM K · Mops (pH 7.4)/5 mM $MgCl_2$/1 mM dithiothreitol, and centrifuged for 15 min in an Eppendorf centrifuge. The latter step was repeated once and the final membrane pellet was suspended with a syringe in about 0.3 ml of the same solution.

Ca^{2+} uptake measurements. The incubation medium contained 150 mM KCl, 20 mM K · Mops (pH 7.4), 5 mM $MgCl_2$, 4 mM Mg · ATP, 50 μM $^{45}CaCl_2$ (10 μCi/ml), 2.5 mM ouabain, 10 μM oligomycin and 0.2–0.3 mg/ml of membrane protein. The uptake reaction was started by the addition of 20 μl of membrane suspension to 200 μl of the incubation medium at 37 °C. At given time intervals, 20 μl samples were withdrawn and filtered through cellulose nitrate filters (0.5 μm pore diameter). The filters were washed twice with 4 ml portions of ice-cold 'stop solution', which contained 150 mM KCl/20 mM K · Mops (pH 7.4)/2 mM EGTA. Radioactivity retained on the filters was measured by scintillation counting.

The kinetics of Ca^{2+} transport were obtained from the measurements of initial rates of Ca^{2+} uptake in calcium buffers [18]. The incubation media contained 100 mM KCl/50 mM K · Mops (pH 7.4)/5 mM $MgCl_2$/0.2 mM HEDTA/0.3–0.5 mM EGTA/0.30 mM $CaCl_2$ (15 μCi/ml)/10 μM oligomycin. Where indicated, bovine brain calmodulin, at concentrations given in the tables, was also included. Free Ca^{2+} concentrations were calculated by a computerized iterative method as described before [17]. 20 μl of BLM suspension were added to 100 μl of the incubation medium and the mixture was preincubated for 4 min at 37 °C. At time 0, 5 μl of 100 mM Mg · ATP was rapidly mixed into the medium. After 15, 45, 75 and 105 s 20 μl samples were withdrawn, filtered and washed. The uptake data were plotted and checked for linearity, and the initial rates of Ca^{2+} uptake ($V_0[Ca^{2+}]$) were calculated as the slopes of the regression lines of Ca^{2+} uptake versus time. At high Ca^{2+} uptake rates, the 105 s time-points often deviated from linearity and were disregarded.

Marker enzymes. Na^+/K^+-ATPase activity was measured by an automated coupled enzyme assay [19] in the presence and in the absence of 2.5 mM ouabain. The activities of aminopeptidase M, γ-glutamyltransferase and alkaline phosphatase were measured kinetically as described before [19]. Protein was measured by the method of Bradford [20] after solubilizing the membranes in 0.25 M NaOH/0.1% (w/v) Triton X-100, which allowed elimination of the interference of Percoll that might still be present in membrane suspensions [21].

Results

Membrane isolation and marker enzyme activities

The kidneys of pre-hypertensive MHS rats are smaller than those of MNS controls [22], which raised the possibility that the kidney cortex plasma membranes might show different behavior during isolation, either due to the presence of different cell populations in kidney cortex or due to different properties of the plasma membranes themselves [23]. Therefore, the BLM isolation procedure was reexamined and compared in the two strains. The results are shown in Table I and Fig. 1. It was confirmed that the kidneys of MHS rats were significantly smaller than those of MNS controls. Protein content and the specific activities of

TABLE I

MARKER ENZYME ACTIVITIES IN THE WHOLE KIDNEY, OUTER KIDNEY CORTEX AND ISOLATED BASOLATERAL PLASMA MEMBRANES

The data are means ± S.E. from three independent experiments. * $P < 0.05$ by paired t-test.

	Control	MHS
Whole kidney		
Weight (mg/kidney)	661 ± 83	545 ± 69 *
Protein (mg/g tissue)	257 ± 30	251 ± 20
Na^+/K^+-ATPase (mU/mg protein)	72 ± 7	67 ± 8
Aminopeptidase M (mU/mg protein)	85 ± 7	73 ± 6 *
γ-glutamyltransferase (mU/mg protein)	401 ± 68	440 ± 46
Outer kidney cortex		
Na^+/K^+-ATPase (mU/mg protein)	59 ± 7	62 ± 8
Aminopeptidase M (mU/mg protein)	88 ± 5	72 ± 6 *
γ-Glutamyltransferase (mU/mg protein)	521 ± 69	586 ± 58
Isolated BLM		
Na^+/K^+-ATPase (mU/mg protein)	3047 ± 243	3220 ± 296
enrichment	53	49
Aminopeptidase M (mU/mg protein)	62 ± 7	53 ± 7 *
enrichment	0.7	0.7

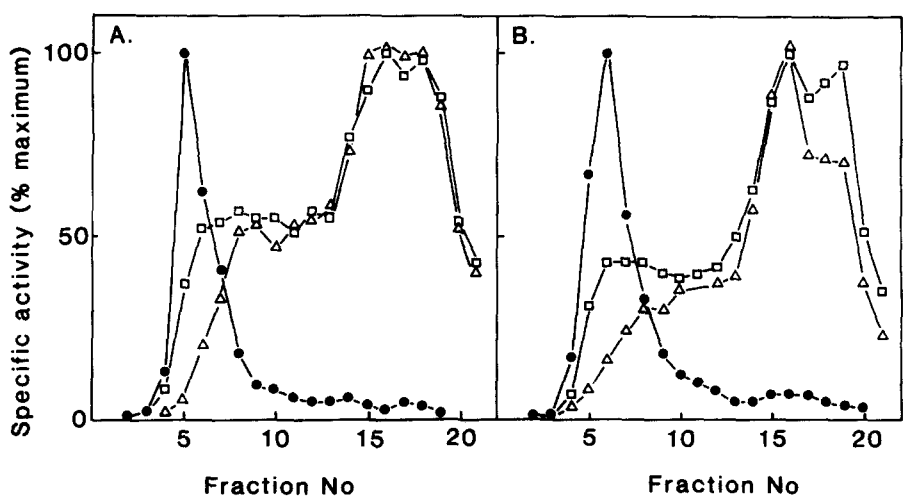

Fig. 1. Distribution of plasma membrane marker enzymes on Percoll gradients. (A) Plasma membranes from control MNS rats; (B) plasma membranes from pre-hypertensive MHS rats. ●, Na^+/K^+-ATPase; △, aminopeptidase M; □, γ-glutamyltransferase. Mean values from quadruplicate determinations in a single representative experiment are shown.

the BLM marker Na^+/K^+-ATPase and of the brush-border membrane marker γ-glutamyltransferase were the same in both whole kidney homogenates and outer kidney cortex homogenates of the two strains (Table I). The specific activity of another brush-border membrane marker, aminopeptidase M, was significantly lower in both whole kidneys and in kidney cortex of MHS rats than in MNS controls. The analysis of membrane marker distribution on Percoll gradients has shown that both the position of Na^+/K^+-ATPase peaks, indicating the density of BLM, and the specific activities of Na^+/K^+-ATPase in peak fractions were the same in the two strains. The distribution of brush-border membrane markers was broad, with peaks in fractions 15 and 18. The distribution of γ-glutamyltransferase (Fig. 1) and of alkaline phosphatase (not shown) was similar in the two strains. The activities of aminopeptidase M, however, were significantly lower in heavy fractions of brush-border membranes of MHS rats. These data suggest that the renal basolateral membranes of MHS rats do not differ from those of MNS controls to the extent that would affect their separation on density gradients. The kidneys of pre-hypertensive MHS rats contain brush-border membranes which have normal γ-glutamyltransferase and alkaline phosphatase activities, but a considerably decreased activity of aminopeptidase M. This defect may have possible consequences for the metabolism of peptides (e.g., hormones) in the renal tubules of MHS rats [24].

ATP-dependent Ca^{2+} uptake

The general pattern of ATP-dependent Ca^{2+} uptake, which is an expression of the Ca^{2+}-transporting ATPase activity [15,18] in BLM vesicles from pre-hypertensive MHS rats and MNS controls, is shown in Fig. 2. In the presence of ATP, the BLM vesicles accumulated Ca^{2+} to levels exceeding 7–10-fold those observed either in the absence of ATP (not shown) or in the presence of Ca^{2+} ionophore A23187. Partial replacement of K^+ in the medium with Na^+ (ouabain being present already at the freezing step, and then throughout) results in a considerable reduction of ATP-dependent Ca^{2+} uptake, which indicates the presence of Na^+–Ca^{2+} exchange in the same membranes as those that contain the ATP-dependent Ca^{2+} pump [15,16,18,25]. The qualitative pattern of ATP-dependent Ca^{2+} transport is quite similar in MHS and MNS strains, except that the levels of Ca^{2+} accumulation are slightly lower (16%, $P < 0.01$, $n = 4$) in membranes from MHS rats.

The activity of Na^+–Ca^{2+} exchange is difficult to measure directly in kidney BLM preparations because of the low degree of vesiculation and high

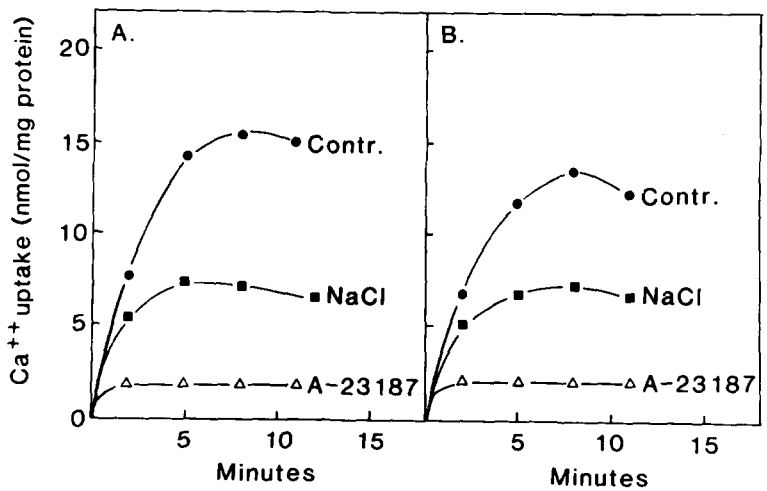

Fig. 2. General characteristics of ATP-dependent Ca^{2+} transport in BLM vesicles from control MNS (A) and pre-hypertensive MHS rats (B). Where indicated, KCl in the medium was replaced with 115 mM NaCl. Ca^{2+} ionophore A23187 concentration was 2 μM. Mean values from quintuplicate determinations in a single experiment are shown.

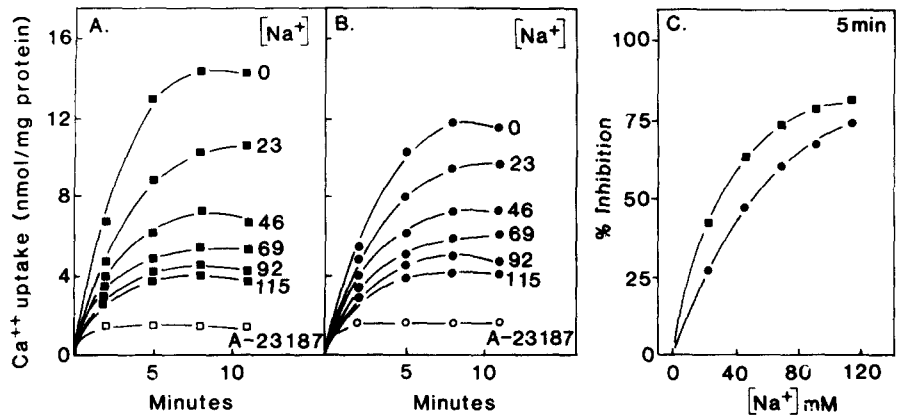

Fig. 3. Effects of Na^+ on ATP-dependent Ca^{2+} uptake in BLM from control MNS (A) and pre-hypertensive MHS rats (B). KCl in the incubation medium was replaced with the NaCl concentrations (mM) shown in the figure. The incubation time was 5 min. The data are means from quadruplicate determinations (S.D. < 5%) in a single representative experiment. ■, control MNS BLM; ●, pre-hypertensive MHS BLM.

permeability to Na^+ [15,25,26]. It can be, however, evaluated indirectly from the inhibition by Na^+ of ATP-dependent Ca^{2+} transport (Ref. 15, Fig. 2).

The effects of increasing concentrations of Na^+ on ATP-dependent Ca^{2+} transport in BLM membranes from MHS and MNS rats are shown in Fig. 3. In membranes from both strains, the ATP-dependent Ca^{2+} transport is progressively inhibited with increasing Na^+ concentrations, but higher Na^+ concentrations are required to produce a 50% inhibition in MHS than in MNS rats (48 and 26 mM Na^+, respectively). These results might suggest that the activity of Na^+–Ca^{2+} exchange is lower in BLM from pre-hypertensive MHS rats.

Kinetics of ATP-dependent Ca^{2+} transport

The kinetics of ATP-dependent Ca^{2+} transport in BLM from MHS and MNS rats, in the presence and in the absence of exogenous calmodulin, is

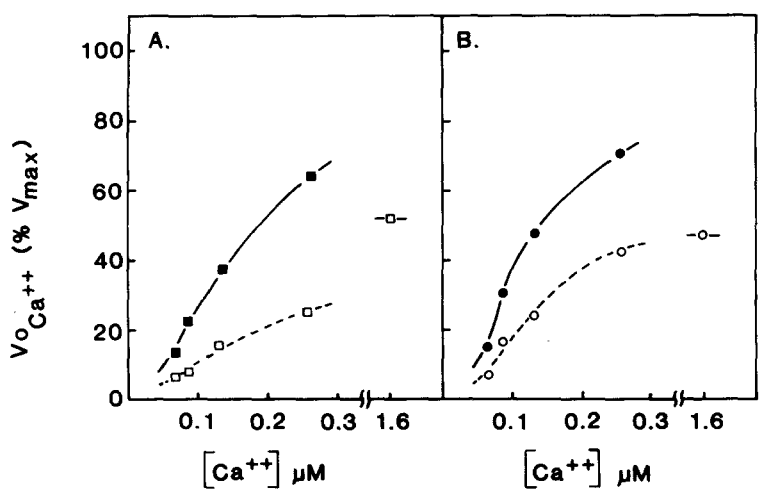

Fig. 4. Ca^{2+} concentration dependence of ATP-driven Ca^{2+} uptake in BLM vesicles from control MNS (A) and pre-hypertensive MHS (B) rats: effects of exogenous calmodulin. Intestinal rates of ATP-dependent Ca^{2+} uptake were measured as described in Materials and Methods. The data are expressed as percent of uptake at 1.6 μM free Ca^{2+} (5.3 and 4.1 nmol/min per mg protein in MNS and MHS, respectively, which were not different from the extrapolated V_{max} values). Open symbols: without calmodulin; filled symbols: 0.3 μM bovine brain calmodulin was present during preincubation and uptake measurements.

shown in Fig. 4. The points represent the initial linear rates of Ca^{2+} uptake (V_0 [Ca^{2+}]), and are expressed as percent of V_{max} to facilitate the comparison between two membranes having different maximal Ca^{2+} transport rates. In control MNS membranes in the absence of exogenous calmodulin, the rates of Ca^{2+} transport are low. Upon addition of exogenous calmodulin, the rates of Ca^{2+} transport increase severalfold and the kinetics become sigmoidal, due to a characteristic positive cooperativity in the Ca^{2+}-ATPase-calmodulin system [27,28]. In BLM from pre-hypertensive MHS rats, the rates of Ca^{2+} transport at low Ca^{2+} concentrations are higher than in control and the kinetics are sigmoidal; addition of exogenous calmodulin results in a further stimulation of Ca^{2+} uptake rates, while the kinetics remain sigmoidal (Fig. 4). The different kinetic behavior of Ca^{2+} transport in BLM from the two strains is illustrated in the Hill plots (Fig. 5). In BLM from control MNS rats, the slope (n) of the Hill plot of ATP-dependent Ca^{2+} transport is about 1 in the absence and about 2 in the presence of exogenous calmodulin. In BLM from pre-hypertensive MHS rats, the slopes are about 2 both in the absence and in the presence of added calmodulin. The kinetic constants of ATP-dependent Ca^{2+} uptake

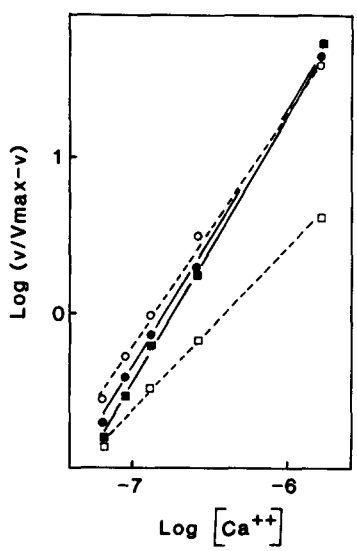

Fig. 5. Hill plots of the kinetic data shown in Fig. 6. □, ■, control MNS BLM; ○, ●, pre-hypertensive MHS BLM; open symbols: without exogenous calmodulin; filled symbols: 0.3 μM bovine brain calmodulin added.

TABLE II

KINETICS OF ATP-DEPENDENT Ca^{2+} UPTAKE IN RENAL BASOLATERAL PLASMA MEMBRANES FROM PRE-HYPERTENSIVE MHS RATS AND THEIR AGE-MATCHED CONTROLS: EFFECTS OF EXOGENOUS CALMODULIN

The data are means ± S.E. from four independent experiments, performed in quadruplicates. The K_m and V_{max} values were derived from plots of $1/v$ vs. $1/[S]^n$, where n = Hill coefficient. The plots were in all cases linear ($r^2 > 0.99$). Bovine brain calmodulin concentration was 0.2 μM. * $P < 0.05$ by paired t-test.

	Without calmodulin	With calmodulin
Control MNS rats		
$K_m(Ca^{2+})$ (μM)	0.24 ± 0.07	0.16 ± 0.02 *
V_{max} (nmol/min per mg protein)	4.55 ± 1.52	5.50 ± 1.84 *
n_{Hill}	0.97 ± 0.15	1.83 ± 0.06 *
Pre-hypertensive MHS rats		
$K_m(Ca^{2+})$	0.13 ± 0.03	0.13 ± 0.03
V_{max} (nmol/min per mg protein)	3.42 ± 0.85	4.62 ± 1.42 *
n_{Hill}	1.92 ± 0.14	2.19 ± 0.13

in BLM from MNS and MHS rats are summarized in Table II. In control MNS membranes, the response of Ca^{2+} transport to exogenous calmodulin is typical [15,17,18,27,28]: a decrease of $K_m(Ca^{2+})$, an increase of V_{max} and an increase of the Hill coefficient from about 1 to about 2. In BLM from MHS rats, on the other hand, the $K_m(Ca^{2+})$ is already low (0.13 μM), and it is not decreased further by the addition of calmodulin. The Hill coefficient is about 2 both before and after calmodulin addition. The V_{max}, however, is significantly increased by exogenous calmodulin. This latter result is rather surprising, since it is generally assumed that the Ca^{2+}-calmodulin complex activates the Ca^{2+}-ATPase via a mixed-type affinity-velocity effect, i.e., it decreases the $K_m(Ca^{2+})$ and increases the V_{max} simultaneously [27–32].

Kinetics of Ca^{2+} transport in BLM extracted with salt and EDTA

The results presented above suggested that in BLM from pre-hypertensive MHS rats, the Ca^{2+}-pumping ATPase existed mostly in an activated, high-affinity state. This might result either from incomplete removal of endogenous calmodulin (al-

TABLE III

KINETICS OF ATP-DEPENDENT Ca^{2+} UPTAKE IN RENAL BLM EXTRACTED WITH EDTA AND KCl

The kinetic data are derived from a representative experiment similar to that shown in Fig. 4, except that the membranes were extracted with hypotonic EDTA and hypertonic KCl (see Materials and Methods). All measurements were done in quadruplicate (S.D. < 5%), and kinetic plots were linear ($r^2 > 0.99$). Bovine brain calmodulin concentration was 0.2 μM.

	Without calmodulin	With calmodulin
Control MNS rats		
$K_m(Ca^{2+})$ (μM)	0.58	0.13
V_{max} (nmol/mg×min)	4.36	4.62
n_{Hill}	1.14	1.93
Pre-hypertensive MHS rats		
$K_m(Ca^{2+})$ (μM)	0.47	0.15
V_{max} (nmol/mg×min)	3.48	4.09
n_{Hill}	0.93	1.80

though the membranes from MHS and MNS rats were handled in exactly the same way), or from the activation of the enzyme by some other mechanism(s), e.g., by acidic phospholipids, free fatty acids or partial proteolysis [31–33]. To distinguish between these possibilities, the membranes were subjected to a more drastic extraction procedure, which involved osmotic shock in the presence of EDTA (to break the vesicles and to remove all divalent cations) and incubation at a high KCl concentration (to weaken protein–protein interactions). The results are summarized in Table III. In BLM from pre-hypertensive MHS rats, the application of this extraction procedure resulted in a 'normalization' of Ca^{2+} transport kinetics. In the absence of exogenous calmodulin, the Ca^{2+} concentration dependence of Ca^{2+} transport was now hyperbolic, with a Hill coefficient of about 1 in both MHS and MNS rats. The addition of exogenous calmodulin resulted in a decrease of $K_m(Ca^{2+})$, an increase of V_{max} and an increase of the Hill coefficient to about 2 in membranes from both strains. The only difference that remained was a slightly lower V_{max} of Ca^{2+} transport in BLM from MHS rats.

Calmodulin concentration dependence of Ca^{2+} transport

The observation that in BLM from pre-hypertensive rats the Ca^{2+}-transporting ATPase was not deactivated by freezing and washing in the presence of EGTA, but could be deactivated by extraction with EDTA and high salt, suggested that calmodulin might be more strongly bound to BLM from pre-hypertensive MHS rats, i.e., the affinity for calmodulin might be increased. To test this possibility, we measured the calmodulin concentration dependence of Ca^{2+} transport in BLM that had been extracted with EDTA and salt. The results are shown in Fig. 6. The calmodulin activation curve is indeed shifted slightly to the left in BLM from pre-hypertensive MHS rats, but difference is not statistically significant. In separate experiments, the activation of Ca^{2+} transport by calmodulin was either clearly higher in BLM from MHS rats (three out of seven experiments) or not different at all (four experiments). The reasons for these inconsistencies are not clear, but they might suggest that the interaction between Ca^{2+}-ATPase and calmodulin is influenced by some factor(s) which could not be sufficiently controlled in the present experiments. This suggestion might be supported by recent observations in red blood

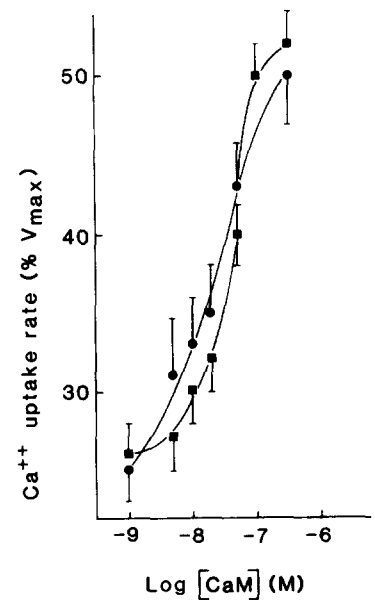

Fig. 6. Calmodulin concentration dependence of Ca^{2+} transport in BLM from control MNS and pre-hypertensive MHS rats. Initial rates of ATP-dependent Ca^{2+} uptake were measured in the presence of bovine brain calmodulin at concentrations shown on the ordinate. ■, control MNS BLM; ●, pre-hypertensive MHS BLM. Ca^{2+} concentration was 0.12 μM.

TABLE IV

KINETICS OF ATP-DEPENDENT Ca^{2+} UPTAKE IN RENAL BLM FROM OLD (14–18 WEEKS) RATS. THE MEMBRANES WERE EXTRACTED WITH EDTA AND KCl

The kinetic data were calculated from one of three similar experiments, with measurements done in quadruplicate (S.D. < 5%). Bovine brain calmodulin was 0.2 μM.

	Without calmodulin	With calmodulin
Control MNS rats		
$K_m(Ca^{2+})$ (μM)	0.20	0.20
V_{max} (nmol/mg×min)	6.08	7.60
n_{Hill}	2.18	1.93
Hypertensive MHS rats		
$K_m(Ca^{2+})$ (μM)	0.23	0.24
V_{max} (nmol/mg×min)	5.56	6.72
n_{Hill}	2.00	2.05

cells showing that cytosolic proteins from MHS rats stimulate the erythrocyte Ca^{2+}-ATPase to a higher degree than either the same fraction from MNS rats or pure calmodulin [34].

Kinetics of Ca^{2+} transport in old rats

Some kinetic experiments were also performed in BLM from mature (14–18 weeks) MHS rats with fully developed hypertension and in their age-matched MNS controls. The results are shown in Table IV. Despite extraction of the membranes with EDTA and salt, the Ca^{2+} transport shows high-affinity cooperative kinetics in both control MNS and hypertensive MHS rats. These results suggest that in older rats, the Ca^{2+}-transporting ATPase in renal BLM becomes irreversibly activated.

Discussion

The calmodulin-dependent, Ca^{2+}-transporting ATPase located in the basolateral plasma membranes of renal tubular cells plays an important role in Ca^{2+} extrusion from the cell and in the regulation of cell Ca^{2+} (for reviews, see Refs. 14, 15). Another Ca^{2+}-transporting system present in these membranes, the Na^+–Ca^{2+} exchange, is much less active, and it probably mediates the Ca^{2+} influx into the cell [14,35]. The reversible, Ca^{2+}-dependent activation of the Ca^{2+}-ATPase by calmodulin is considered to be of primary importance for the regulation of the cytosolic Ca^{2+} concentration (for a review, see Ref. 27). Due to activation by calmodulin, the Ca^{2+}-ATPase can switch from an almost inactive to an almost fully activated state within the relatively narrow range of Ca^{2+} concentrations that may occur in the cell.

In the BLM of pre-hypertensive MHS rats, the V_{max} of Ca^{2+}-transporting ATPase is about 16% lower than in MNS controls. More significantly, the pattern of activation of the Ca^{2+}-ATPase by calmodulin is different in the two strains. In BLM from control MNS rats, isolated in the presence of EGTA, the Ca^{2+}-ATPase is present mostly in a low-affinity, low-velocity state and it responds to exogenous calmodulin with a decrease of $K_m(Ca^{2+})$, an increase of V_{max} and an increase of Hill coefficient from about 1 to about 2. This is a typical behavior characteristic for a deactivated enzyme depleted of calmodulin [15–18,27,28]. In BLM from pre-hypertensive MHS rats the $K_m(Ca^{2+})$ of the Ca^{2+}-ATPase is already low and it is not decreased further by exogenous calmodulin. The hill coefficient is about 2 in the absence of added calmodulin (Table II). These data suggest that in BLM from MHS rats, the Ca^{2+}-ATPase is present mostly in an activated state. A slight increase of the V_{max} upon addition of exogenous calmodulin suggests that a small proportion of enzyme molecules are in a deactivated state: their activation by calmodulin does not visibly change their general kinetic characteristics, but the total activity is increased. The activation of Ca^{2+}-ATPase in pre-hypertensive MHS membranes can be reversed only under drastic conditions: incubation in hypotonic EDTA and then in hypertonic KCl (neither of which alone is effective). This observation seems to exclude the acidic phospholipids or the partial proteolysis as activating mechanisms, since incubation in EDTA and KCl is not likely to remove lipids from the membrane, and prolonged incubations would be expected to increase rather than prevent proteolysis.

Enhanced activation of the Ca^{2+}-ATPase in pre-hypertensive MHS rats might result from its increased affinity for calmodulin. Unfortunately, the experiments designed to test this hypothesis did not provide unequivocal results (Fig. 6). In some experiments, an increased sensitivity of the

Ca^{2+}-ATPase to calmodulin was indeed observed, but in others, no difference could be seen. These results might suggest that increased affinity for calmodulin, if present, is not an inherent property of the Ca^{2+}-ATPase, but results from some other reactions which could not be controlled in our experiments.

Our data also suggest that the deactivation of the Ca^{2+}-ATPase is not a simple matter of lowering the Ca^{2+} concentration in the medium, which should result in a dissociation of calmodulin from the enzyme. Obviously, in BLM from pre-hypertensive rats, the activation of Ca^{2+}-ATPase is not readily reversible as in MNS controls, which might imply some other regulatory mechanism(s). This conclusion would be in line with the observation (Table IV) that in old rats of both strains, the Ca^{2+}-ATPase in renal plasma membranes is irreversibly activated. The mechanisms involved in both cases might be different, but nevertheless these data suggest that freely reversible activation of plasma membrane Ca^{2+}-ATPase by calmodulin may not be a universal phenomenon.

The Ca^{2+}-ATPase is a dominant transport system involved in Ca^{2+} exit from renal tubular cells [14,15]. It seems reasonable to assume that abnormal regulation of this enzyme may affect the regulation of the cellular Ca^{2+} concentration. Direct interpretation of in vitro kinetic data in terms of Ca^{2+} fluxes in the cell is obviously impossible. However, it may be hypothesized that enhanced activation of Ca^{2+}-ATPase in the basolateral plasma membranes of pre-hypertensive MHS rats might result in a higher rate of Ca^{2+} transport out of the cell and in a decrease of cytosolic Ca^{2+} concentration in resting cells. Cytosolic Ca^{2+} is a major regulator of transepithelial Na^+ transport which decreases the permeability of the brush-border membranes to Na^+. At lower cytosolic Ca^{2+}, the permeability of the brush-border membranes to Na^+ can be expected to increase. This has indeed been observed in brush-border membranes from pre-hypertensive MHS rats [11]. Taken together, these data suggest that enhanced activation of Ca^{2+}-ATPase in renal basolateral plasma membranes of pre-hypertensive MHS rats may contribute to the enhanced Na^+ reabsorption and Na^+ retention, which is characteristic of several types of essential hypertension.

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