Decreased Calcium Pump Activity in Duodenal Epithelial Cells from Spontaneously Hypertensive Rats (43620)

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Abstract. **Recently, hypertension research has focused on altered cytosolic free Ca^{2+}, $[Ca^{2+}]_i$, in cells of spontaneously hypertensive rats (SHR). In this work, a mechanism(s) responsible for regulating $[Ca^{2+}]_i$ was studied with duodenal epithelial cells isolated from SHR and their control, normotensive Wistar-Kyoto rats (WKY). The equal specific activity of sucrase, an enzyme characteristic of microvilli, in both mucosal scrapings and isolated cells from SHR and WKY suggested that mucosal density of enterocytes was not largely different between the two strains. $[Ca^{2+}]_i$ of the isolated cells was estimated with fura-2. Upon incubation in the presence of $CaCl_2$, $[Ca^{2+}]_i$ increased to a larger extent in SHR than in WKY cells. Ca^{2+} efflux based on measurements of $[Ca^{2+}]_i$ was decreased in SHR cells. Correspondingly, it was found that ^{45}Ca efflux at 10 sec was lower for SHR cells than for WKY cells. Specific activities of Ca^{2+}-stimulated and Ca^{2+}/calmodulin-stimulated ATPases in basolateral plasma membrane preparations were reduced in SHR cells. These results indicated that Ca^{2+} efflux was decreased by reduction in Ca^{2+}-pumping ATPase activity in SHR enterocytes.**

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The spontaneously hypertensive rat (SHR) is a model used in the study of human essential hypertension. So far, a number of biochemical and physiologic defects related to calcium metabolism of SHR have been reported. More recently, hypertension research has focused on alterations in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Elevated $[Ca^{2+}]_i$ has been found in various tissues obtained from SHR.

It is generally accepted that luminal Ca^{2+} enters the intestinal cell across the brush border membrane along its electrochemical gradient via facilitated diffusion. On the other hand, the ATP-driven Ca^{2+} transport and Na^+-Ca^{2+} antiport in the basolateral membrane (BLM) are assumed to be responsible for extrusion of Ca^{2+}. Various techniques have been utilized for studying Ca^{2+} transport in the intestine of SHR and have yielded conflicting findings (1–3).

Ca^{2+} transport by duodenal sacs of SHR was reported previously to be decreased (2). However, it was later shown that Ca^{2+} uptake by brush border membrane vesicles isolated from SHR was not reduced (4). In contrast, an increased Ca^{2+} uptake was found for the same membrane vesicles of SHR (5). On the other hand, there have been reports that Ca^{2+} uptake by both duodenal enterocytes (6) and brush border membrane vesicles (7) of SHR was significantly decreased.

Ca^{2+} efflux was found to be decreased in duodenal enterocytes (8) and BLM vesicles (9) isolated from SHR. Na^+-Ca^{2+} antiport activity was also reduced in the BLM of SHR enterocytes, while ATP-dependent Ca^{2+} transport was not changed (10). These findings were so conflicting that it was thought worthwhile to estimate $[Ca^{2+}]_i$ and to study its possible changes in the duodenal cells isolated from SHR and Wistar-Kyoto rats (WKY). No evidence has so far been presented to indicate possible $[Ca^{2+}]_i$ changes in intestinal epithelial cells of hypertensive animals. Notably, little is known about Ca^{2+}/calmodulin (CaM)-dependent ATPase activity in intestinal cells from hypertensives.

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The purpose of the present work was to estimate $[Ca^{2+}]_i$ of duodenal absorptive cells isolated from SHR and to demonstrate a possible Ca^{2+} extrusion abnormality in SHR cells involving Ca^{2+}/CaM-dependent ATPase activity.

Materials and Methods

Materials. ^{45}Ca (370 MBq/mg of Ca) was obtained from New England Nuclear and bovine testis CaM was obtained from Biomedical Technologies, Inc. Fura-2 acetoxymethyl ester was purchased from Dojindo Laboratories (Tokyo).

Animals. Male SHR and WKY of the Okamoto-Aoki strain, 8 weeks of age, were purchased from Shizuoka Laboratory Animal Center (Hamamatsu). Each strain was kept on a 12:12-hr light:dark cycle and allowed free access to pelleted chow containing 1.6% Ca, 1.2% P, 0.4% Mg, 0.2% Na, 2.0 IU/g of vitamin D_3, and water. Blood pressure was measured by a tail cuff and a pneumatic pulse transducer connected to a recorder. Systolic blood pressure of SHR (155 ± 3 mm Hg) was significantly ($P < 0.01$) greater than that of WKY (115 ± 3 mm Hg) at 12 weeks.

Cell Preparation. Animals, 12–14 weeks of age, were sacrificed by cervical dislocation and the duodenum (10-cm-long segment adjacent to the pylorus) was rapidly removed, flushed, and chilled in ice-cold 0.9% NaCl containing 1 m*M* dithiothreitol. Villus cells were isolated by a modified method of Weiser (11). The duodenum was everted and distended by filling with phosphate-buffered saline containing 1.5 m*M* EDTA and 0.5 m*M* dithiothreitol (pH 7.4) (Solution B). The everted duodenum was incubated for 10 min at 37°C in phosphate-buffered saline containing 27 m*M* citrate (pH 7.4) (Solution A). The everted sac was then incubated for 7 min in Solution B. The loosened cells were washed off by repeatedly removing and reimmersing the specimen in Solution B. Villus cells were suspended in 250 m*M* mannitol containing 3 m*M* KH_2PO_4, 1 m*M* $MgCl_2$, 5 m*M* NaCl, 0.5 m*M* β-hydroxybutyrate, 2.5 m*M* glutamine, 10 m*M* mannose, 1 mg/ml of bovine serum albumin, and 20 m*M* HEPES (pH 7.4). The final content of cell protein was 1.5–2.0 mg/ml. Cell viability was tested by the trypan blue extrusion method. More than 90% of the cells isolated from the two strains were equally viable. As described below, isolated cells were loaded with fura-2 and washed twice. The density of the cells was adjusted and stored in the dark at room temperature. The total time consumed for these procedures was about 1 hr.

Membrane Preparation. Basolateral plasma membranes of SHR and WKY enterocytes were prepared as described previously (12). Usually, five rats were sacrificed for one preparation. The final membrane fraction was washed twice with hypoosmotic EDTA solution containing 20 m*M* HEPES and 5 m*M* EDTA (pH 7.4), to be deprived of endogenous CaM (13).

Estimation of $[Ca^{2+}]_i$. Duodenal epithelial cells were loaded with 5 μ*M* fura-2 acetoxymethyl ester in the medium consisting of 140 m*M* KCl, 10 m*M* HEPES (pH 7.4), 2.5 m*M* glutamine, and 10 m*M* mannose (Medium A) as described previously (14). Briefly, after loading at 37°C for 15 min, the cells were washed twice with Medium A. The density of the cells was adjusted to $1–3 \times 10^6$ cells/ml. An aliquot of 100 μl of the cell suspension was added to 1.9 ml of the prewarmed Medium A in a cuvette and the mixture was stirred continuously at 37°C. Fluorescence of fura-2 was measured with a Cairn spectrophotometer system and $[Ca^{2+}]_i$ was estimated based on the fluorescence ratio, as described previously (14). Leakage of the dye was negligible since an addition of EGTA to the final concentration of 300 μ*M* induced no rapid reduction in the fluorescence ratio.

Ca^{2+} Uptake and Extrusion Estimated with Fura-2. The cells were loaded with 5 μ*M* fura-2 and washed as described above. An aliquot of 100 μl of the loaded cells was added to 1.9 ml of Medium A at 37°C in a cuvette. Ca^{2+} uptake was initiated by addition of $CaCl_2$ dissolved in Medium A to the final concentration of 200 μ*M*. An increase in $[Ca^{2+}]_i$ followed as described above. To examine the Ca^{2+} extrusion from SHR and WKY cells, these cells were first loaded with fura-2 and then with Ca^{2+} in the presence of 200 μ*M* $CaCl_2$, as described above. After 3 min of incubation, EGTA that had been dissolved in Medium A (pH 7.4) was added to the final concentration of 300 μ*M* and the decrease of $[Ca^{2+}]_i$ was followed fluorometrically.

^{45}Ca Efflux Measurement. The method of measuring ^{45}Ca efflux from the enterocytes was similar to that employed by Hanai *et al.* (15). Cell suspension (100 μl) was preloaded with calcium by adding to 100 μl of the incubation medium containing 140 m*M* KCl, 10 m*M* HEPES, 2 m*M* $Ca(^{45}Ca)Cl_2$ (18.5 kBq) (pH 7.4) and incubating the mixture under constant shaking at 37°C for 20 min. Na^+ was excluded from the reaction medium as mentioned above. The Ca^{2+}-loaded cells (200 μl) were rapidly mixed with 2 ml of the prewarmed EGTA solution containing 140 m*M* KCl, 10 m*M* HEPES, and 2 m*M* EGTA (pH 7.4). At an appropriate time, the efflux was terminated by the addition of 4 ml of precooled EGTA solution and the mixture was filtered rapidly on a 5-μm Millipore filter (SMWP 02500). The filter was washed three times with 2 ml of the precooled EGTA solution and counted for radioactivity with a scintillation counter. Zero time values (0% efflux) were estimated from reactions in which the precooled EGTA solution was added before the efflux medium and the contents of the reaction tube were filtered immediately.

Ca^{2+}-ATPase Assay. To assay Ca^{2+}-ATPase, a 10-

μl aliquot of a basolateral membrane fraction (10–20 μg of protein) was added to 0.6 ml of the medium containing 100 m*M* KCl, 5 m*M* $MgCl_2$, 0.5 m*M* EGTA, 5 m*M* theophylline, 2 m*M* ouabain, 3 m*M* Na_2 ATP, 100 μg/ml of oligomycin, and 20 m*M* HEPES-Tris (pH 7.4). The incubation lasted for 30 min at 37°C. Reaction was terminated by adding 0.1 ml of 20% sodium dodecyl sulfate and liberated inorganic phosphate was measured by the method of Fiske and Subbarow [16]. Basal Ca^{2+}-ATPase activity was calculated by subtracting the activity obtained with 0.5 m*M* EGTA alone from that in the presence of 1 μ*M* free Ca^{2+}. Ca^{2+}/CaM-stimulated ATPase activity was estimated by subtracting the activity of basal Ca^{2+}-ATPase from that in the presence of 0.6 μ*M* CaM in addition. The activities were expressed as μmol released Pi mg protein^{-1} hr^{-1}. The total $CaCl_2$ concentrations were estimated as described previously (14).

Sucrase and Na^+,K^+-ATPase Assay. The activities of sucrase and Na^+,K^+-ATPase were estimated as described previously (12).

Data Analysis. All data are presented as means ± SE. Significant differences between means were determined using either Student's *t* test, for one variable, or analysis of variance, for multiple variables, followed by Scheffe's multiple range test. Differences were considered statistically significant at $P < 0.05$.

Results

Ultrastructural characteristics of the duodenal cells isolated from SHR and WKY were examined by high resolution scanning electron microscopy. No differences were found in the density of the microvilli between the two strains in disagreement with the previous finding of "patchy loss of microvilli" on the surface of SHR cells.

In order to know whether or not the duodenal cell preparations isolated from SHR and WKY had the same density of absorptive cells, the specific activity of sucrase, a marker enzyme for the brush border, was determined for mucosal scrapings and isolated cells (12). As presented in Table I, it was not different between the two strains.

The $[Ca^{2+}]_i$ of the isolated cells was estimated with fura-2 as described in Materials and Methods. Basal $[Ca^{2+}]_i$ of the freshly prepared SHR and WKY cells were 58 ± 3 n*M* and 53 ± 4 n*M*, respectively (Fig. 1A). The $[Ca^{2+}]_i$ increased during incubation of the cells with 200 μ*M* $CaCl_2$. The increase was greater for SHR than for WKY cells at 7 min of incubation (Fig. 1B). The increment of $[Ca^{2+}]_i$ was 72 ± 6 n*M* for SHR and 55 ± 5 n*M* for WKY cells.

As Ca^{2+} efflux could be responsible for regulation of $[Ca^{2+}]_i$ in these cells, it was studied by fura-2 fluorometry (Fig. 2A). For this purpose, the duodenal cell suspensions were preloaded with Ca^{2+} ions in the presence of 200 μ*M* $CaCl_2$ at 37°C for 3 min. The $[Ca^{2+}]_i$ was not statistically different between the two strains at 3 min of Ca^{2+} preloading: 91 ± 8 n*M* for SHR and 86 ± 5 n*M* for WKY cells (Fig. 1B). Ca^{2+} extrusion was initiated by adding EGTA to the final concentration of 300 μ*M*. The rate of $[Ca^{2+}]_i$ decrease at 10 and 60 sec is presented in Figure 2B. Ca^{2+} effluxes based on measurements of $[Ca^{2+}]_i$ decrements at 10 and 60 sec were 21.2 ± 2.1% vs 30.8 ± 2.2% ($P < 0.01$) and 39.3 ± 3.5% vs 51.3 ± 4.8% ($P < 0.05$) original $[Ca^{2+}]_i$ for the SHR vs WKY cells, respectively.

^{45}Ca efflux was measured to determine whether or not the decrease in $[Ca^{2+}]_i$ after the addition of EGTA reflected the Ca^{2+} efflux. No significant difference was observed in ^{45}Ca preloading at 5 min between SHR and WKY cells (4.21 ± 0.45 and 4.07 ± 0.38 nmol/mg protein, respectively [$n = 8$ pairs]). Figure 3 shows the

Table I. Sucrase Activity of Mucosal Scrapings and Villus Cells Isolated from SHR and WKY Rats[a]

Preparations	SHR ($n = 10$)	WKY ($n = 10$)
Mucosal scrapings	4.12 ± 0.44	4.28 ± 0.43
Isolated cells	4.05 ± 0.34	4.07 ± 0.38

[a] Sucrase was assayed as described previously (12). Activities are in micromole of glucose liberated mg protein^{-1} hr^{-1} (means ± SE).

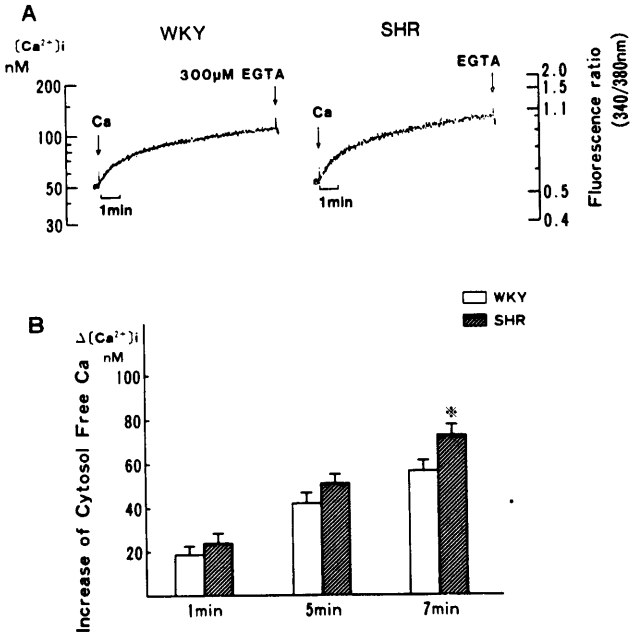

Figure 1. Ca^{2+} uptake by isolated duodenal cells estimated by increase of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). Enterocytes were loaded with 5 μ*M* fura-2 in the medium consisting of 140 m*M* KCl, 10 *mM* HEPES (pH 7.4), 2.5 m*M* glutamine, and 10 m*M* mannose. Ca^{2+} uptake was initiated by addition of $CaCl_2$ to the final concentration of 200 μ*M*. $[Ca^{2+}]_i$ was estimated as described in Materials and Methods. (A) Representative tracings of Ca^{2+} uptake with fura-2 loaded cells. (B) Mean $[Ca^{2+}]_i$ increase from basal values of each strain ($n = 12$ pairs). $P < 0.05$ for comparison between groups.

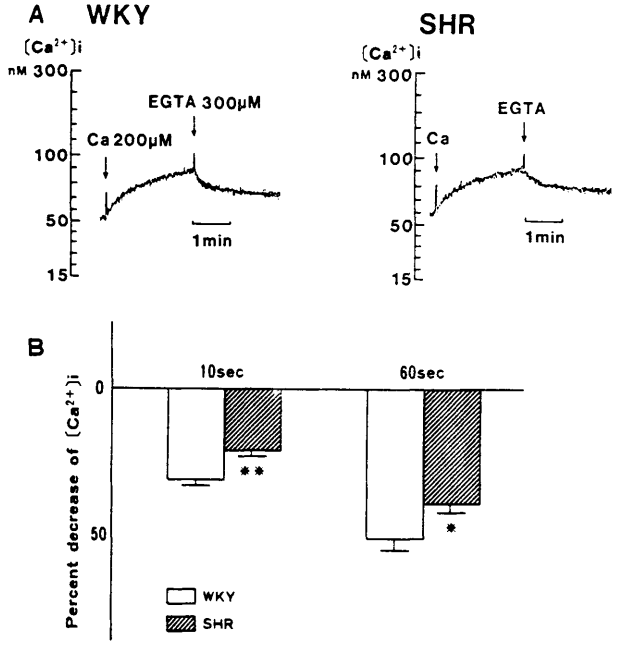

Figure 2. Decrease in $[Ca^{2+}]_i$ after the addition of EGTA to the incubation milieu containing Ca^{2+} preloaded cells. The fura-2 loaded cells were incubated in the presence of 200 μM $CaCl_2$ for 3 min as described in the Legend to Figure 1. (A) The extrusion of Ca^{2+} was initiated by addition of EGTA to the final concentration of 300 μM $[Ca^{2+}]_i$ after 3 min of incubation with 200 μM $CaCl_2$ (final) was 91 ± 8 nM and 86 ± 5 nM in SHR and WKY cells, respectively. (B) $[Ca^{2+}]_i$ decrease at 10 and 60 sec after the addition of EGTA is expressed in percentage of original $[Ca^{2+}]_i$. $P < 0.05$ and $P < 0.01$ for comparisons between groups.

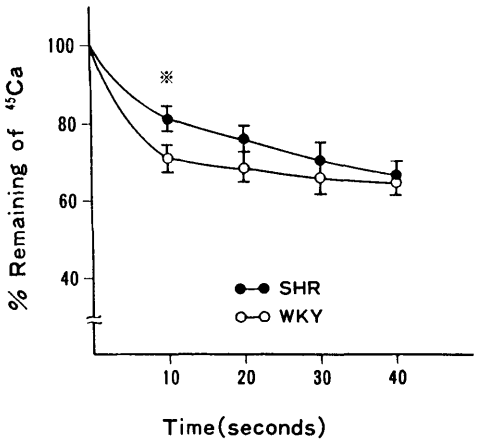

Figure 3. ^{45}Ca efflux from isolated enterocytes. Isolated cells were preloaded in the presence of 1 mM $Ca(^{45}Ca)Cl_2$ as described in the text and ^{45}Ca efflux initiated by the dilution of the cell suspension with a medium containing 2 mM EGTA. ^{45}Ca efflux is expressed as the percentage of tracer remaining in duodenal cells. Each datum represents the mean ± SE of eight experiments for each cell preparation. Every experiment was carried out in triplicate. $P < 0.05$ for comparison between groups.

time course of ^{45}Ca efflux from these cells in the presence of EGTA (see Materials and Methods for details). Na^+ was not included in the reaction medium as stated above. ^{45}Ca efflux at 10 sec was significantly ($P < 0.05$) decreased in SHR enterocytes.

It is established that high affinity Ca^{2+}-ATPase (Ca^{2+}-pump) is located in the BLM of enterocytes. However, little is known about its activity in the plasma membrane of SHR cells. In the present work, therefore, the BLM was prepared from SHR and WKY duodenal mucosa as described in Materials and Methods. Na^+,K^+-ATPase activity (μmol/mg protein/hr) was 2.36 ± 0.38 and 53.6, respectively, for the homogenate and the basolateral membrane vesicles from SHR cells (23-fold enrichment) and 4.06 ± 0.46 and 82.4 ± 9.5 for those from WKY cells (20-fold enrichment). The Mg^{2+}-ATPase activity of these membranes was 8.28 ± 0.92 for SHR and 9.72 ± 1.96 μmol hr^{-1} mg $protein^{-1}$ for WKY cells (Table II). The Ca^{2+}-stimulated ATPase activity was estimated in the presence of 0.1–100 μM Ca^{2+}. The optimal stimulation was obtained at 1.0–10 μM for both strains (data not shown). The ATPase activity in the presence of 1 μM free Ca^{2+} and that in the presence of 1 μM Ca^{2+}/0.6 μM CaM were both reduced in SHR (Table II). In a preliminary study, Ca^{2+}/CaM-stimulated ATPase was assayed with a final CaM concentration at 0.2–1.4 μM in the presence of 1 μM free Ca^{2+}. CaM induced a dose-dependent increase in the enzyme activity of the BLM and the maximum activity was attained at 0.6 μM for both strains (data not shown). On the other hand, the Mg^{2+}-ATPase activity was not affected by CaM for either strain.

Discussion

An attempt to estimate $[Ca^{2+}]_i$ of villus cell suspensions with quin-2 has been unsuccessful. However, $[Ca^{2+}]_i$ of ileal cells was successfully estimated with fura-2 (14). A preliminary study showed that fura-2/AM was available for estimating $[Ca^{2+}]_i$ in duodenal epithelial cells. To the best of our knowledge, $[Ca^{2+}]_i$ of SHR enterocytes has been determined and its change studied for the first time in the present work.

The basal $[Ca^{2+}]_i$ estimated in this study was somewhat lower than that reported previously for enterocytes (17). In light of the fact that $[Ca^{2+}]_i$ in erythrocytes varies with the extracellular Ca^{2+} concentration (18),

Table II. Mg^{2+}-ATPase, Ca^{2+}-ATPase, and Ca^{2+}/CaM-ATPase of BLM Isolated from SHR and WKY Rat Enterocytes[a]

ATPases	SHR ($n = 6$)	WKY ($n = 6$)	P
Mg^{2+}-ATPase	8.28 ± 0.92	9.72 ± 1.96	NS
Ca^{2+}-ATPase	0.98 ± 0.06	1.65 ± 0.18	<0.01
Ca^{2+}/CaM-ATPase	0.63 ± 0.09	0.96 ± 0.13	<0.05

[a] Ca^{2+}-ATPase activity was calculated by subtracting the activity obtained with EGTA alone from that in the presence of 1 μM Ca^{2+}. Ca^{2+}/CaM-ATPase was estimated by subtracting the activity obtained with Ca^{2+}-ATPase from that in the presence of 0.6 μM CaM in addition to 1 μM Ca^{2+}. Activities are in micromole of liberated P_i (means ± SE).

the low $[Ca^{2+}]_i$ of the SHR and WKY enterocytes isolated in the present work might be due to the low extracellular Ca^{2+} concentration.

Two distinct mechanisms are presumably involved in the extrusion of Ca^{2+} from the cell: an Na^+-Ca^{2+} antiport system and an active transport system (Ca^{2+}/CaM-ATPase). The present work showed that Ca^{2+} efflux measured with fura-2 or isotopic Ca^{2+} was decreased in SHR enterocytes in the absence of the extracellular Na^+ ions. Evidence to date suggests an abnormality in the Ca^{2+}-ATPase activity in platelets (19) and erythrocytes (20) of SHR. Actually, it has been suggested that $[Ca^{2+}]_i$ might be elevated in SHR as a result of reduced Ca^{2+}-pump activity (19). More recently, it is suggested that the reduction in the intestinal Ca^{2+} absorption in SHR is not due to a decrease in Ca^{2+} influx at the brush border membrane, but due to that in Ca^{2+} efflux at the BLM (5). Our result is compatible with a previous studyon the disturbance of ATP-dependent Ca^{2+} uptake in BLM vesicles of SHR (9), even though some controversy remains (8, 10), probably due to differences in experimental design. Shibata and Ghishan (10) did not find a significant difference in the ATP-driven transport of Ca^{2+} by BLM vesicles between the two strains. However, the results of a Ca^{2+} transport experiment performed with BLM vesicles should be interpreted with caution because, as shown previously, a BLM preparation is usually contaminated by endoplasmic reticulum membrane vesicles (21). Although it was reported that Ca^{2+} efflux of the duodenal enterocytes without Na^+ did not differ between 5 and 60 min (8), we found that ^{45}Ca efflux at 10 sec was significantly decreased in SHR (Fig. 3). If the Ca^{2+} efflux observed in the present work was the result of an increased permeability of the isolated cells, it would be difficult to explain its observed difference between the two strains when the specific activity of sucrase and the uptake of L-alanine and 3-*O*-methyl-D-glucose were not different between the two strains (unpublished work). Our results suggest that the decreased Ca^{2+} efflux may probably result from the reduced activity of Ca^{2+}-pump in SHR enterocytes. The decrease in Ca^{2+}-pump activity in SHR might be due to (i) decreased intracellular ATP, (ii) decreased CaM, and (iii) impaired Ca^{2+}-pump activity, e.g., reduced number of transporters.

A patchy loss of microvilli detected by Drueke *et al.* (22) was not detected on the SHR cell in the present work. This discrepancy might be due to the difference of the method. A possibility could not be ruled out that the scanning electron microscopy failed to detect patchy defects of microvilli, especially when the size of a patch was very small compared with the height of microvilli. The previous authors have noted the normal length of them adjacent to such a patch.

CaM-regulated Ca^{2+}-transporting ATPase in the plasma membrane has been assumed to be involved in maintaining low $[Ca^{2+}]_i$ in intact cell (23). Intestinal Ca^{2+}-ATPase exhibits enzymic characteristics similar to those of erythrocyte Ca^{2+}-ATPase (24). Abnormal Ca^{2+}-ATPase activity has been reported previously for the SHR erythrocytes and platelets, whereas only one report has been published on the decreased Ca^{2+}/CaM-ATPase activity in the intestine of SHR (25). In the present study, both basal and Ca^{2+}/CaM-ATPase activities were decreased in the duodenal BLM of SHR. Equal enrichment of Na^+,K^+-ATPase in the duodenal BLM preparations of two strains suggested that the difference in Ca^{2+}-ATPase activity was not due to a difference in purification. At present, it is not known whether this diminished Ca^{2+}-ATPase activity is a result of reduced expression of the protein, intrinsic defect in the enzyme itself, or altered microenvironment in the membrane. More detailed characterization is needed to substantiate these speculations.

To the best of our knowledge, this is the first report on the intracellular Ca^{2+} concentration of isolated enterocytes in SHR and WKY. A possibility was suggested that Ca^{2+} efflux was decreased by reduction in Ca^{2+}-transporting ATPase activities in SHR enterocytes. These findings are compatible with decreased transepithelial calcium transport in SHR (2, 26). Whether this abnormality is a primary event or a result of hypertension remains to be resolved.

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