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Increase in Na^+-Ca^{2+} Exchange Activity in Sarcolemma Isolated from Mesenteric Arteries of Spontaneously Hypertensive Rats

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Abstract□Na^+-Ca^{2+} exchange process in sarcolemmal vesicles isolated from mesenteric arteries of Wistar-Kyoto normotensive(WKY) and spontaneously hypertensive rats(SHR) was investigated. The sarcolemmal fractions isolated after homogenization and sucrose density gradient centrifugation were enriched with 5'-nucleotidase and ouabain sensitive, K^+-dependent phosphatase activities. When the vesicles were loaded with Na^+, a time dependent Ca^{2+} uptake was observed. However, very little Ca^{2+} uptake was observed when the vesicles were loaded with K^+, or Ca^{2+} uptake of the Na^+-loaded vesicles was carried out in high sodium medium so that there was no sodium gradient. When the vesicles loaded with Ca^{2+} by Na^+-Ca^{2+} exchange were diluted into potassium medium containing EGTA, Ca^{2+} was rapidly released from the vesicles. Na^+-dependent Ca^{2+} uptake was increased in SHR compared to WKY, but passive efflux of preaccumulated Ca^{2+} from the vesicles was decreased in SHR. The data indicate that the membrane vesicles of rat mesenteric arteries exhibit Na^+-Ca^{2+} exchange activity. It is also suggested that changes of this process in vascular smooth muscle cell membrane of SHR may be involved in higher intracellular Ca^{2+} concentration and higher basal tone in SHR.

Keywords□Na^+-Ca^{2+} exchange, sarcolemma, SHR, mesenteric artery.

Despite the widely recognized correlation between sodium intake and hypertension in hypertensive animals and man,[1-5] the underlying mechanism of the development of hypertension has not been elucidated. However, a number of papers have reported that membrane abnormalities including alternations in cation transports[6-10] and calcium handling[11-14] play an important role in this mechanism. Since intracellular calcium is a main determinant for contraction of vascular smooth muscle[15-17] and intracellular sodium plays a critical role in the maintenance of calcium homeostasis in smooth muscle[18], changes in calcium and sodium regulation could be a plausible cause of increase of vascular smooth muscle tone in hypertension. Indeed, it has been suggested that the altered reactivity of smooth muscle fiber in the veins of spontaneously hypertensive rats (SHR) is consequence of elevated intracellular calcium ion[19] and that increased sensitivity to norepinephrine and potassium and decreased rate of relaxation of the SHR aortas are due to a decrease in the rate of ATP dependent calcium efflux from the smooth muscle cell.[20] Also, increase in Ca^{2+}-uptake of duodenum[13] and cardiac sarcolemma[21] in SHR was reported. In addition to these reports, it has been shown that Na^+-pump activity is decreased in experimentally induced hypertensive animals[22-25] and SHR.[26] Na^+-pump inhibition would lead to partial depolarization of cell membrane and thus increases vascular reactivity. An intriguing speculation is that inhibition of the Na^+, K^+-ATPase results in elevated intracellular Na^+ which, in turn, would diminish the sodium gradient for calcium extrusion and/or increase Na^+-Ca^{2+} exchange.[27] Therefore, inhibition of Na^+-pump activity in SHR might be an important cause of increase of vascular tone. However, studies of Na^+-Ca^{2+} exchange in isolated vascular smooth muscle membrane from SHR have not performed. In the present study, Na^+-Ca^{2+} exchange in sarcolemmal vesicles isolated from mesenteric arteries of normotensive (WKY) rat and SHR was examined.

EXPERIMENTAL METHODS

Animals

4-5 months old spontaneously hypertensive rats

(SHR) and control normotensive Wistar-Kyoto (WKY) rats were used. Systolic blood pressure was determined with the tail of prewarmed, unanesthetized rats by the tail-cuff method.[28] The rats were killed by a blow on the head and the mesenteric vascular bed including mesenteries, fat, veins, arteries was rapidly removed and placed in freshly prepared ice-cold 0.25 M sucrose solution containing 5 mM Tris-HCl, pH 7.4 (Medium A). After separation of mesenteric artery, 15 to 20 arteries were pooled to prepare membrane fraction.

Preparation of membrane fraction

The arteries isolated from the whole vascular bed were placed in a 25 m*l* of Medium A and minced with a pair of scissors, and homogenized four times with a Sorvall omnimixer for 15 seconds each. The homogenate was centrifuged at 1,500 × g for 10 min. The supernatant was centrifuged at 27,000 × g for 20 min to remove heavier mitochondrial and other membrane fractions as pellets. The supernatant was then centrifuged at 100,000 × g for 60 min to sediment a microsomal fraction. The pellet was gently homogenized in medium A and applied to the top of a 24% (w/v) discontinuous sucrose gradient. The gradient tube was centrifuged for 30 min at 73,400 × g. The middle white layer between medium A and 24% sucrose solution was carefully removed from the gradient tube using pasteur pipettes and diluted with Na^+-buffer containing 160 mM NaCl, 20 mM Mops/Tris, pH 7.4. The suspension was centrifuged at 73,400 × g fir 30 min to yield a final pellets which were used to prepare uniform suspensions of Na^+-loaded vesicle.

Protein content was estimated by the method of Lowry *et al.*[29] using bovine serum albumin as a standard.

Measurement of Ca^{2+}-uptake

To measure Ca^{2+}-uptake, sarcolemmal vesicles were preloaded with Na^+ by resuspension in 160 mM NaCl and 20 mM MOPS/Tris (pH 7.4) (Na^+-buffer). 2 u*l* (3-4 ug) of the Na^+-loaded vesicles were added to a series of tubes containing 100 u*l* of Na^+ or K^+ buffer and 10 μM $^{45}CaCl_2$. Na^+-dependent Ca^{2+}-uptake was terminated by the addition of 5 m*l* of ice-cold washing solution containing 200 mM KCl, 0.1 mM EGTA, 5 mM MOPS/Tris (pH 7.4), and the vesicles were rapidly harvested on Millipore filters (0.45 um, Gelman) by filtration. The filters were washed twice with 5 m*l* of washing solution and then dissolved in 8 m*l* of a scintillation liquid containing 12 g of 2.5-diphenyloxazole, 450 mg of 1.4-bis-[2-(5-phenyloxazolyl)]-benzene, 1*l* of Triton X-100 and 2 *l* of toluene. The radioactivity retained on the filters was counted by liquid scintillation counter. Vesicles preloaded with 160 mM KCl and 20 mM MOPS/Tris (pH 7.4) (K^+-buffer) were used as blanks to estimate nonspecific binding or uptake.

Measurement of Ca^{2+}-efflux

The sarcolemmal vesicle were first loaded with Ca^{2+} by diluting 0.5 μ*l* of the Na^+-loaded vesicle into 15 μ*l* of 160 mM KCl, 40 μM $^{45}CaCl_2$, 20 mM MOPS/Tris (pH 7.4). This mixture was incubated for 3 min at 37 °C. At 3 min the Ca^{2+}-loaded vesicles were diluted a second time to stop influx and to initiate efflux by the addition of 1 m*l* of efflux medium containing K^+-buffer and 0.1 mM EGTA. The content of Ca^{2+} within the vesicles after various times of efflux was determined by filtration of the mixture through a 0.45 μm Millipore filter under suction. The filter was then washed three times with 5 m*l* of washing solution as mentioned above and the quantity of ^{45}Ca remaining in the filtered vesicles was determined by liquid scintillation counter.

Enzyme assays

5 '-Nucleotidase was assayed at 37 °C in 100 mM Tris-Cl buffer (pH 7.4) containing 10 mM $MgCl_2$ using 5 '-AMP as substrate.[30] Inorganic phosphate released during 15 min reaction time was determined according to the method of Fiske and Subbarow.[31] K^+-dependent phosphatase activity was measured in the presence of 10 mM KCl, 10 mM $MgCl_2$, 8 mM p-nitrophenyl phosphate, 50 mM L-histidine buffer (pH 7.4) and membrane protein in the presence or absence of 1 mM ouabain at 37 °C for 15 min. Ouabain sensitive phosphatase activity was determined as the difference in activity in the presence and absence of 1 mM ouabain.

RESULTS

Marker enzyme activity of homogenate and sarcolemmal fraction

The mean body weight and systolic blood pressure of the SHR and WKY are given in Table I. The total body weights of the SHR were the same as for WKY. The blood pressures of the SHR were significantly increased over those of the WKY controls.

Table I. Body weights and blood pressure of rats

	Number of rats	Body weight (g)	Blood pressure (mmHg)
Normotensive rats(WKY)	50	265 ± 8	122 ± 4
Spontaneously hypertensive rats(SHR)	50	272 ± 7	186 ± 5*

Determinations of body weight and blood pressure were made one day prior to sacrifice. Values are mean ± SEM.
* Significantly different from corresponding value of WKY.

Table II. 5′-Nucleotidase and ouabain-sensitive, K-dependent phosphatase activities of homogenate and plasma membrane from normotensive rat mesenteric arteries

Fraction	Enzyme activity*	
	5′-nucleotidase (μmol pi/mg/hr)	K^+-phosphatase (μmol p-nitrophenol/mg/hr)
Homogenate	6.2 ± 0.6	0.27 ± 0.09
Plasma membrane	42.1 ± 3.2	2.53 ± 0.34

* Measurement of 5′-nucleotidase and K^+-dependent phosphatase activities was carried out as described in Method. Values are mean ± SEM of three different preparations of normotensive rats.

The enzyme activities of the fractions from rat mesenteric arteries are shown in Table II. Sarcolemmal fraction exhibited high specific activity of sarcolemma marker enzymes (5′-Nucleotidase and ouabain sensitive, K^+-dependent phosphatase). These membrane markers were enriched about 7 to 10 fold in the sarcolemmal fraction compared to the starting homogenate.

Na^+-induced Ca^{2+}-uptake

In order to demonstrate Na^+-Ca^{2+} exchange in the sarcolemmal vesicles of mesenteric arteries, the vesicles were loaded internally with 160 mM NaCl which allowed Na^+ to enter by passive diffusion.

These Na^+-loaded vesicles were then diluted 1:50 into 160 mM KCl containing 10 μM $^{45}CaCl_2$. Under these conditions, a rapid uptake of $^{45}Ca^{2+}$ was observed which reached maximum at 3 min (Fig. 1). However, $^{45}Ca^{2+}$ uptake of the vesicles preloaded with 160 mM KCl was only 10% of that of the Na^+-loaded vesicles under the same conditions. When the Na^+-loaded vesicles were diluted into 160 mM NaCl (containing $^{45}Ca^{2+}$) so that there was no sodium gradient, $^{45}Ca^{2+}$-uptake was not observed (Table III). Therefore, Na^+-Ca^{2+} exchange of vesicles was corrected for the Ca^{2+}-uptake that occurred in the same experiments performed using K^+-loaded vesicles.

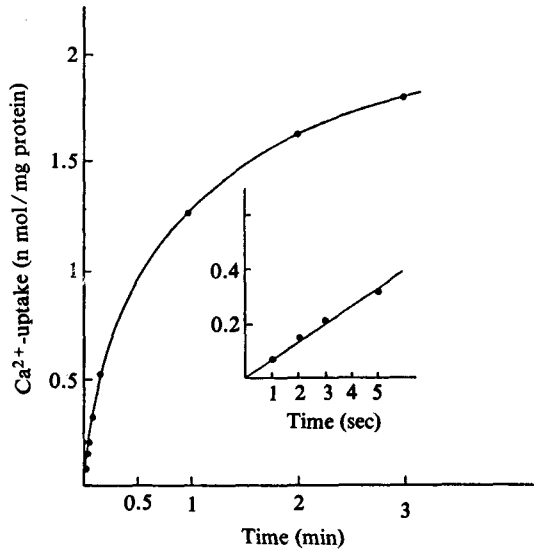

Fig. 1. Time course of Ca^{2+}-uptake by Na^+-loaded vesicles from mesenteric arteries of normotensive rats.
Vescles were preloaded in 160 mM NaCl, 20 mM MOPS/Tris at pH 7.4. At t = 0, 2 μl of Na^+-loaded vesicles were rapidly added to 100 μl of 160 mM KCl, 20 mM MOPS/Tris, pH 7.4 at 37 °C containing 10 μM ^{45}Ca. All points are corrected for any Ca^{2+} uptake which occurred when the reaction was begun using K^+-loaded vesicles. For further details, see Methods. Each point denotes the mean of Ca^{2+}-uptake obtained with three different preparations.

The time course of Na^+-Ca^{2+} exchange presented in Fig. 1 shows that the maximal Na^+-dependent Ca^{2+}-uptake was about 1.8 n mol/mg protein, and initial rate was 0.06 n mol/mg protein per sec.

The linearity of Ca^{2+}-uptake was maintained to about 5 seconds. Fig. 2 demonstrates Na^+-dependent Ca^{2+}-uptake of the Na^+-loaded vesicles prepared from mesenteric arteries of WKY and SHR. The vesicles from SHR accumulated about 2.5 n mol/mg protein of Ca^{2+} at 3 min, indicating about 1.4-fold increase in SHR relative to WKY. As shown in inset, the initial rate of Ca^{2+}-uptake was also increased in SHR.

Table III. Ca^{2+} uptake by Na^+-loaded and K^+-loaded vesicles

Uptake medium	$^{45}Ca^{2+}$ uptake (n mol/mg protein)	
	Na^+-loaded	K^+-loaded
160 mM KCl	2.0 ±0.24	0.18±0.03
160 mM NaCl	0.13±0.02	0.12±0.02

Vesicles were preloaded in either 160 mM NaCl, 20 mM MOPS/Tris at pH 7.4 (Na^+-loaded) or 160 mM KCl, 20 mM MOPS/Tris at pH 7.4 (K^+-loaded), and then added to 100 μl of 160 mM KCl or 160 mM NaCl, 20 mM MOPS/Tris, pH 7.4 at 37°C containing 10 μM $^{45}Ca^{2+}$. The reaction was stopped at 3 min by the rapid addition of 5 ml of 200 mM KCl, 0.1 mM EGTA. 5 mM MOPS/Tris, pH 7.4, followed by Millipore filtration as described in Method. Values are mean ± SEM of three different preparations from WKY.

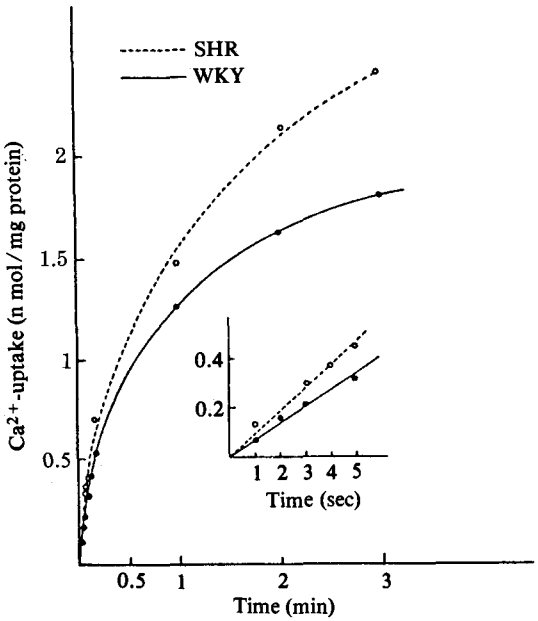

Fig. 2. Time course of Ca^{2+} uptake by Na^+-loaded vesicles from mesenteric arteries of normotensive (WKY) and hypertensive (SHR) rats.
Na^+-dependent Ca^{2+}-uptake of the vesicles was measured as described in Fig. 1 and Methods.

Ca^{2+}-efflux from the vesicles

The rate of Ca^{2+} efflux from the vesicles was examined. In this experiment, Na^+-loaded vesicles were allowed to accumulate $^{45}Ca^{2+}$ for 3 min by Na^+-Ca^{2+} exchange at pH 7.4. After 3 min of Ca^{2+}-uptake (When a plateau of uptake was obtained), the vesicles were diluted 65.5-fold into K^+-buffer containing EGTA (to inhibit any further

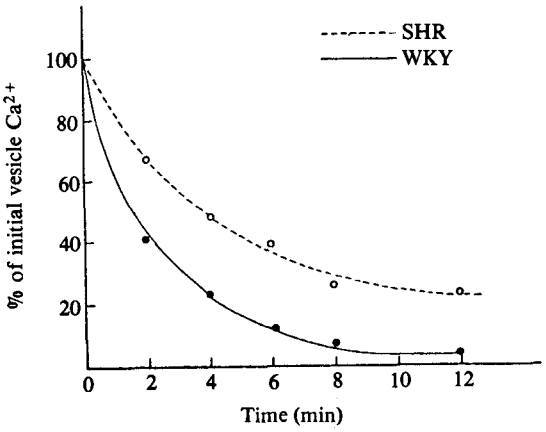

Fig. 3. Ca^{2+}-efflux from sarcolemmal vesicles prepared from mesenteric arteries of WKY and SHR.
The $^{45}Ca^{2+}$-loaded vesicles were prepared by Na^+-Ca^{2+} exchange by diluting 0.5 μl of Na^+-loaded vesicles(or K^+-loaded vesicles as blanks) into 15 μl of 160 mM KCl, 40 μM $^{45}CaCl_2$, 20 mM MOPS/Tris (pH 7.4, at 37 °C). After 3 mins, 1 ml of efflux medium containing 160 mM KCl, 0.1 mM EGTA and 20 mM MOPS/Tris at pH 7.4 was added. The data are expressed as the percentage of Ca^+ remaining within sarcolemmal vesicles after given efflux time. The inital Ca^{2+}-loads of three different preparations from mesenteric arteries of WKY and SHR were 1.82 ± 0.21 n mol/mg protein and 2.51 ± 0.28 n mol/mg protein, respectively. Other legends are the same as described in Fig. 1 and Method.

Ca^{2+}-uptake), and intravesicular Ca^{2+} content was measured at various fixed times by Millipore filtration. Any remaining externally bound Ca^{2+} was corrected by use of appropriate blanks.

As shown in Fig. 3, the Ca^{2+}-loaded vesicles exhibited the passive efflux of Ca^{2+} and half time of $^{45}Ca^{2+}$ efflux from the Ca^{2+}-loaded vesicles was about 1.5 min. $^{45}Ca^{2+}$-efflux from the sarcolemmal vesicles of WKY mesenteric arteries were compared to that of SHR mesenteric arteries in Fig. 3. There was significant difference in the rate of Ca^{2+}-efflux between WKY and SHR. The amount of Ca^{2+}-efflux from the vesicles of SHR decreased at all the time point.

DISCUSSION

The results presented in this study demonstrate that a Na^+-Ca^{2+} exchange process exists in the plasma membrane of rat mesenteric arteries and that this process is increased in SHR.

The interrelationship between Na^+ and Ca^{2+} in

regulation of intracellular free Ca^{2+} and the maintenance of cardiac contractility has been mainly explained by a sarcolemmal Na^+-Ca^{2+} exchange mechanism. Since Reeves and Sutko[32] identified Na^+-Ca^{2+} exchange activity in isolated cardiac sarcolemmal vesicles, several reports on the properties of sarcolemmal Na^+-Ca^{2+} exchange have appeared in cardiac[33-35] and skeletal muscle membrane.[36] This transport system is electrogenic[33,34] and a specific property of sarcolemma.[32,33,37] However, there is no direct evidence that Na^+-Ca^{2+} exchange occurs across vascular smooth muscle cell membrane. In this study, Na^+-Ca^{2+} exchange in the sarcolemmal vesicles isolated from mesenteric arteries of rats was investigated.

The sarcolemmal vesicles isolated from mesenteric arteries accumulated Ca^{2+} rapidly to a steady state level of 1.8 n mol/mg protein at an external Ca^{2+} concentration of 10μM when an outwardly directed Na^+ gradient was generated across the membrane vesicles. When the vesicles were loaded with K^+ or when the Na^+-loaded vesicles were added to 160 mM NaCl containing $^{45}Ca^{2+}$, $^{45}Ca^{2+}$-uptake was not observed. These results indicate that a sodium gradient is necessary for Ca^{2+} uptake and sarcolemmal vesicles derived from rat mesenteric arteries like those from cardiac and skeletal muscle are capable of Na^+-Ca^{2+} exchange.

The observed Na^+-Ca^{2+} exchange is a sarcolemmal activity. This is supported by the correlation between Na^+-dependent Ca^{2+}-uptake and 5'-nucleotidase or K^+-dependent phosphatase activity which was enriched about 7-to 10-fold in sarcolemmal fractions seperated by sucrose density gradient centrifugation compared to the starting homogenate. The possibility that the observed Na^+-Ca^{2+} exchange activity was due to mitochondrial or sarcoplasmic reticulum contamination was excluded by the following observations. First, Na^+-Ca^{2+} exchange of mitochondrial fraction isolated from rat mesenteric arteries was examined by the same method as shown in sarcolemmal Na^+-Ca^{2+} exchange but no significant Ca^{2+}-uptake was observed. Second, there was an inverse correlation between azide-sensitive mitochondrial ATPase activity and Na^+-dependent Ca^{2+}-uptake. Third, ATP-dependent Ca^{2+}-uptake of sarcolemmal fraction was not affected by oxalate in contrast to the well-known oxalate-dependent Ca^{2+} uptake in sarcoplasmic reticulum vesicles.

It is generally recognized that several forms of hypertension are associated with an increased peripheral resistance due to maintained abnormal constriction of the small resistance vessels.[38] Since calcium is the primary regulator of contraction in various types of muscles including myocardium and peripheral vasculature, changes in intracellular free Ca^{2+} reflect changes in vascular smooth muscle tone and a rise in the mean intracellular Ca^{2+} may be the final common path by which most hypertension is produced. This assumption has been supported by the reports that decreased rate of relaxation of 6-month-old SHR aortas may be due to decreased calcium extrusion by the cell membrane[20] and the altered reactivity of smooth muscle fibers in the veins of spontaneously hypertensive rats is a consequence of elevated intracellular Ca^{2+}.[19]

The increased intracellular Ca^{2+} concentration in SHR vascular smooth muscle could be accounted for by a reduced ATP-dependent Ca^{2+}-pump or by a increased Na^+-Ca^{2+} exchange activity of the smooth muscle cell membrane. Several investigators[20,39,40] have reported that the sarcolema enriched fraction and microsomes prepared from SHR vascular smooth muscle exhibited a reduced ATP-dependent Ca^{2+} transport when compared with that of preparations from normotensive rats. These reports are consistent with the hypothesis that the slower rate of relaxation of the SHR aortas is due to a decrease in the rate of ATP-dependent Ca^{2+} efflux from the vascular smooth muscle cell membrane. However, it is not known whether changes in Na^+-Ca^{2+} exchange across sarcolemma are involved in the increased intracellular Ca^{2+} in SHR smooth muscle.

In this study, Na^+-dependent Ca^{2+}-uptake of the sarcolemmal vesicles isolated from mesenteric arteries was significantly higher in SHR. This increased Na^+-Ca^{2+} exchange activity was identified in the cardiac sarcolemmal vesicles from SHR.[21] Thus, in addition to a decrease in ATP-dependent Ca^{2+} efflex from the cell membrane, an increase in Na^+-Ca^{2+} exchange activity across the cell membrane may play some role in the development or maintenance of hypertension in SHR.

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