Calcium-Induced Calcium Release in EGTA-skinned Aortic Strips from Genetically Hypertensive and Normotensive Rats

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SUMMARY

The release of ^{45}Ca induced by Ca^{2+} was studied in genetically hypertensive (SHR) and normotensive (WKY) rats using EGTA-skinned aortic strips. Strips preloaded with ^{45}Ca (pCa 6.6) were desaturated at 5 mM of EGTA. A slow component of the washout in aorta from SHRs exhibited higher Ca content and a lower rate of Ca leak than that in aorta from WKY rats. This slow component was stimulated during washing with 0.03, 0.3, 1 or 10 μM free Ca^{2+} . The release of ^{45}Ca induced by Ca^{2+} proceeded at similar rates in preparations of the two strains. Compared to WKY aortic strips the stimulated efflux of ^{45}Ca was greater in SHR aortic strips due to the higher Ca content. About half of the release of ^{45}Ca induced by 1 μM free Ca^{2+} during the first 6 minutes of stimulation was blocked by 0.6 mM of ryanodine or 50 μM of ruthenium red, thus identifying the sarcoplasmic reticulum as a source of Ca release. The results suggest that this intracellular storage of Ca in aorta from genetically hypertensive rats is relevant for the generation of high levels of cytosolic Ca^{2+} . (Jpn Heart J 36: 247–257, 1995)

Key words: Genetically hypertensive rats EGTA-skinned aortic strips
 Ca^{2+} -induced Ca^{2+} release Sarcoplasmic reticulum

THE function of systems involved in the regulation of the cytosolic concentration of Ca^{2+} in vascular smooth muscle cells has been the subject of many studies which aimed to determine the cellular mechanism underlying the increase in vascular tone of genetically hypertensive rats. Concerning the Ca channels of the sarcolemma, a higher influx of Ca^{2+} through the receptor operated ones^{1,2)} and similar^{1,3)} or higher effluxes⁴⁾ through the voltage operated ones, have been described in vascular smooth muscle cells from SHRs compared to

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those from WKY rats. On the other hand, the extrusion of cytosolic Ca^{2+} carried out by the Ca^{2+} -ATPase of sarcolemma has been proved to be depressed in genetically hypertensive rats.^{5,6)} Any of these abnormalities leading to high Ca^{2+} levels in the cytosol might directly increase the vascular tone in SHR. Simultaneously, intracellular Ca stores, namely the sarcoplasmic reticulum, could load more Ca^{2+} and then be able to release larger amounts for activation of contraction. An increased Ca uptake has been reported in intact aortic smooth muscle cells from SHR⁷⁾ but the contribution of the SR in determining the vascular tone of SHR has been usually assessed with mechanical studies. Results comparing the contractile response of vessels from genetically hypertensive and normotensive rats to agonists that mobilize Ca^{2+} from intracellular stores, are controversial. Lower^{8,9,10)} and higher^{1,8,11)} contractions in vessels of SHR compared to those in vessels of WKY rats have been reported.

This work was aimed at a more direct assessment of intracellular Ca^{2+} sources in vessels of genetically hypertensive and normotensive rats. Using EGTA-treated aortic strips, ^{45}Ca content, unstimulated ^{45}Ca efflux and Ca^{2+} stimulated ^{45}Ca efflux were determined in both strains. Preparations of SHR are shown to release larger amounts of Ca upon stimulation with micromolar levels of free Ca^{2+} than those of WKY rats.

METHODS

Adult male rats (16–24 weeks) of genetically hypertensive (SHR) and normotensive (WKY) strains, weighing 250–350 g, were used after 3–4 determinations of their systolic pressure in the tail artery by using the plethismographic method. Mean values of 26 rats in each group were 187 ± 3 mmHg for SHR and 133 ± 2 mmHg for WKY. Rats were anesthetized with Na pentobarbital (80 mg/kg body weight⁻¹, i.p.) and the aorta removed, cleaned of connective tissue and washed in ice cold saline. Strips of 3–5 mg were blotted, weighed and attached to a stainless steel hook (0.22 mm diameter). The chemical skinning of plasma membrane was performed by overnight exposure of strips to a solution at 4°C containing 5 mM EGTA, 170 mM K propionate, 2.5 mM MgCl_2 , 2.5 mM Na_2ATP and 10 mM Hepes buffer, pH 7. Similar procedures have been used to skin strips of cardiac tissue.¹²⁾

Unstimulated efflux of Ca^{2+} : Strips treated with EGTA were incubated for 30 minutes in 1 ml of an uptake solution containing (in mM) 1.6 ^{45}Ca -EGTA (radioisotope from New England Nuclear), 3.4 EGTA, 170 K propionate, 2.5 MgCl_2 , 5 Na_2ATP and 10 Hepes buffer, pH 7. The free Ca^{2+} concentration of 0.25 μM was obtained by diluting CaEGTA buffer stocks prepared with known concentrations of CaCO_3 and EGTA, and calculated with the computer program of

Figure 1. ^{45}Ca accumulation at different times of incubation in the uptake solution described in Methods. After the strips were loaded with ^{45}Ca they were washed for 30 minutes in solutions containing $50\ \mu\text{M}$ of ruthenium red and $10\ \text{mM}$ MgCl_2 ; under these conditions the extracellular ^{45}Ca is eliminated and the loss of ^{45}Ca from the intracellular compartment is prevented. * indicates $p < 0.05$.

Fabiato.¹³⁾ Control experiments were performed in order to demonstrate that after 30 minutes of exposure to the above uptake solution the accumulation of ^{45}Ca was saturated in aortic strips from SHR and WKY rats (Figure 1). In the efflux experiments after the period of ^{45}Ca load, the strips were successively immersed in 2 ml of a desaturation solution at the time intervals indicated in the figures. Desaturation solution contained (in mM) 1 or 5 EGTA, 170 K propionate, 1 MgCl_2 , 5 Na_2ATP and 10 Hepes buffer, pH 7. At the end of the washing, the strips were digested by overnight exposure to 1 ml of 1 N NaOH. Radioactivity in samples obtained during washing and in digested tissue was measured in a liquid scintillation counter (Beckman LS-3133P). Decay of ^{45}Ca content during the period of washing was adjusted by non linear regression to a double exponential decay function. From these curves, the values of both extrapolated Ca content at time zero and half time of washing ($T_{1/2}$) of the fast and slow components identified were calculated.

^{45}Ca stimulated efflux of Ca^{2+} : Skinned strips were loaded with ^{45}Ca as described above and then washed with the desaturation solution containing 5 mM EGTA for 20 minutes followed by 12 minutes of washing with desaturation solutions that contained appropriate amounts of Ca-EGTA buffer, thus attaining a final concentration of EGTA of 5 mM, free Ca^{2+} concentrations of 0.03, 0.3, 1 or 10 μM . Four samples at 3 min intervals were collected during the washing with each of these free Ca^{2+} concentrations followed by a return to the initial desaturation solution (5 mM EGTA) for 6 minutes. With values of ^{45}Ca content in the digested tissue and in samples at each time interval, the efflux of ^{45}Ca and the fraction of ^{45}Ca lost per minute (rate coefficient) were calculated under stimu-

lation with the four free Ca^{2+} concentrations assayed. In order to test the sensitivity of ^{45}Ca efflux induced by micromolar concentrations of free Ca^{2+} to agents that specifically block the Ca channels in sarcoplasmic reticulum, the stimulation was performed in the presence of 0.6 mM of ryanodine or 50 μM of ruthenium red.¹⁴⁾ This concentration of ryanodine blocked the SR- Ca^{2+} release channels in rat cardiac homogenates¹⁴⁾ and it is close to the one described to achieve maximal deactivation of the action of ryanodine (1 mM) on skeletal and cardiac SR- Ca^{2+} release channels in SR vesicles.¹⁵⁾

Statistical analysis was performed using the Student's *t* test for independent samples when comparing means of normotensive and hypertensive groups, and the one-way analysis of variance with the Peritz' F test for differences between stimulations of ^{45}Ca efflux in the absence and presence of Ca channels blockers. The level of statistical significance was set at $p < 0.05$.

RESULTS

EGTA-treated aortic strips preloaded with ^{45}Ca for 30 minutes were washed with desaturation solutions containing 1 or 5 mM of EGTA. The work from Stout and Diecke¹⁶⁾ used segments of tail artery from WKY rats, skinned by treating them with 0.1 mg/ml of saponin and 5 mM EGTA for 60 minutes at 20°C. We used protocols of load and washout in our EGTA skinned aortic strips similar to those showing the sensitivity to caffeine and submicromolar free Ca of a slow compartment in the tail artery.¹⁶⁾ Figure 2 shows data of ^{45}Ca content in tissue during the period of washing. The washout of ^{45}Ca was initially fast, corresponding with a compartment that has been associated with extracellular and cytosolic spaces in the saponin-skinned vessels.¹⁶⁾ The half time of washing for this fast component did not differ between hypertensive and normotensive rats. Values were 0.80 ± 0.10 vs 0.84 ± 0.15 min at 1 mM EGTA and 0.71 ± 0.06 vs 0.66 ± 0.04 min at 5 mM EGTA for the WKY and SHR groups, respectively. The slow component in washout curves had values of extrapolated Ca content slightly higher, but without statistical significance, in SHR than in WKY rats at both EGTA concentrations (Table I). Since the half time of washing was shorter in the normotensive strain at 5 mM of EGTA, a greater content of Ca in the hypertensive strain became significant after ten minutes of washing (Figure 2, B). Ca retained by this slow component could be considered as Ca sequestered in SR of skinned smooth muscle cells without the participation of mitochondria, since at 0.25 μM Ca^{2+} in the uptake solution, an azide-sensitive fraction could not be detected in saponin-skinned vessels.¹⁷⁾ Four concentrations of free Ca^{2+} were assayed during the ^{45}Ca washout of strips preloaded with ^{45}Ca at the time when the slow component was being washed. Figure 3 shows the effects of 0.03 (A), 0.3

Figure 2. Ca content in tissue during desaturation of ^{45}Ca preloaded EGTA-treated aortic strips from SHR and WKY rats. Washing solutions contained 1 or 5 mM EGTA. A significantly higher Ca content of the slow component in strips of SHR is evident with 5 mM EGTA. Ca content was lowered by washing with 5 mM EGTA, which suggests that this concentration was necessary to achieve a full buffering of ^{45}Ca efflux from this intracellular compartment in both strains. A similar value was found to control changes in cytosolic free Ca^{2+} of skinned caudal arteries.¹⁴⁾ Values are mean $\pm$ SEM for the number of experiments indicated in Table I.

Table I. Ca Content (nmol.g^{-1} wt) and Half Time of Wash ($T^{1/2}$, min) of the Slow Component Identified in Washouts of ^{45}Ca Loaded Strips from SHR and WKY Rats

	1 mM EGTA		5 mM EGTA	
	Ca content	$T^{1/2}$	Ca content	$T^{1/2}$
WKY	870 ± 90	23 ± 2	790 ± 90	11 ± 0.6
	(n = 4)		(n = 5)	
SHR	1010 ± 40	25 ± 3	840 ± 70	$14 \pm 0.8^*$
	(n = 4)		(n = 5)	

* indicates $p < 0.05$ between values of WKY and SHR groups.

(B), 1 (C) and 10 (D) μM free Ca^{2+} on rate coefficient values of strips from both strains. No differences in the increases in rate coefficient induced by 0.03, 0.3, 1 and 10 μM free Ca^{2+} were detected between the normotensive and hypertensive groups (Figure 3 and Table II). The efflux of ^{45}Ca during stimulation with the different free Ca^{2+} concentrations was greater in strips from SHRs than in strips

Figure 3. Effects of submicromolar and micromolar free Ca^{2+} concentrations on ^{45}Ca efflux from skinned aortic strips of WKY rats ($\circ$) and SHRs ($\bullet$). Tissues were loaded at $0.25 \mu\text{M}$ free $^{45}\text{Ca}^{2+}$ for 30 minutes and desaturated in 5 mM EGTA until 20 minutes, followed by a period of 12 minutes with washing solutions containing 0.03 (A), 0.3 (B), 1 (C) or 10 (D) μM free Ca^{2+} , and then a return to 5 mM EGTA for 6 minutes. The horizontal bars indicate the time interval of stimulation. The increases in rate coefficient induced were Ca concentration dependent (Table II). Values of Ca content previous to the stimulation as well as of efflux of Ca corresponding with these figures are shown in Table III. Points are means $\pm$ SEM of four experiments for each calcium concentration.

Table II. Maximal Increments in Rate Coefficient, Expressed as % of the Value at the 20th Minute of Washing, during Stimulation with Different Free Ca^{2+} Concentrations

$[\text{Ca}^{2+}] \mu\text{M}$	0.03	0.3	1	10
WKY	23 ± 11	69 ± 6	92 ± 18	117 ± 5
SHR	9 ± 6	71 ± 11	90 ± 20	112 ± 14

from WKY rats, providing that the content of Ca was significantly higher in the hypertensive preparations (Table III). At $0.03 \mu\text{M}$ free Ca^{2+} , where statistically significant changes in rate coefficient were not detected, the efflux of ^{45}Ca was higher in SHR than in WKY. Thus, under both unstimulated and stimulated rate of Ca release, the aortic strips from genetically hypertensive rats lose more Ca than those of the normotensive control group.

Table III. Ca Content before Stimulation (nmol.g^{-1} wt) and Ca Efflux for the Whole Period of Stimulation (nmol.g^{-1} wt. 12 min^{-1}) with Different free Ca^{2+} Concentrations

[Ca^{2+}] μM	Ca content		Ca efflux	
	WKY	SHR	WKY	SHR
0.03	159 ± 18	$227 \pm 23^*$	72 ± 6	$96 \pm 6^*$
0.3	183 ± 5	201 ± 9	108 ± 5	119 ± 6
1	158 ± 2	$210 \pm 18^*$	96 ± 3	$122 \pm 8^*$
10	151 ± 18	$230 \pm 8^*$	100 ± 12	$142 \pm 3^*$

* indicates $p < 0.05$ between values of WKY and SHR groups.

Figure 4. Efflux of ^{45}Ca at the time intervals where a free Ca^{2+} concentration of $1 \mu\text{M}$ was applied. The pre and post-stimulation effluxes are not shown. Effluxes induced in the absence and presence of 0.6 mM ryanodine (Ry) or $50 \mu\text{M}$ ruthenium red (RR) by $1 \mu\text{M}$ free Ca^{2+} are compared among them and with the efflux without stimulation. The SR channel blockers suppressed about 40–50% of the Ca efflux induced by Ca^{2+} during the first 6 minutes of stimulation. Bars represent mean values with indication of the SEM for the same number of experiments in WKY and SHR under the different washing conditions: $5 \text{ mM EGTA} = 14$, $1 \mu\text{M}$ free $\text{Ca}^{2+} = 8$, $1 \mu\text{M}$ free Ca^{2+} plus 0.6 mM ryanodine = 6, and $1 \mu\text{M}$ free Ca^{2+} plus $50 \mu\text{M}$ ruthenium red = 5. * indicates $p < 0.05$ stated by the Peritz' F test between unstimulated (black bars) and stimulated efflux of Ca.

Figure 4 shows the effects of ryanodine and ruthenium red on the release of ^{45}Ca induced by $1\ \mu\text{M}$ free Ca^{2+} . Compared with the unstimulated efflux (black bars), during the first 3 minutes the efflux was stimulated by $1\ \mu\text{M}$ free Ca^{2+} only in the absence of SR-Ca channels blockers (open bars). Computing the net releases induced by $1\ \mu\text{M}$ free Ca^{2+} during the first 6 minutes of stimulation they were reduced by 54% for WKY and 43% for SHR in the presence of ryanodine, and by 37% for WKY and 58% for SHR in the presence of ruthenium red. Thus, about half of the Ca efflux induced by $1\ \mu\text{M}$ free Ca^{2+} during this period was through the SR calcium channels.

DISCUSSION

Ca^{2+} -induced Ca^{2+} release is shown in EGTA-treated multifiber preparations of aorta from SHR and WKY rats. In desaturation curves performed on strips preloaded with ^{45}Ca , a slow compartment was able to increase the ^{45}Ca efflux when the washing media contained micromolar concentrations of free Ca^{2+} . The larger amount of Ca released by aortic strips of the hypertensive strain is supported by the high Ca content of this compartment compared with that of the normotensive WKY strain and not by differences in the rate of Ca efflux. About half of the ^{45}Ca efflux induced by $1\ \mu\text{M}$ free Ca^{2+} during the first 6 minutes of stimulation could be blocked by ryanodine or ruthenium red, thus identifying the sarcoplasmic reticulum as a source of the ^{45}Ca release. Several studies reported that the skinning of muscle fibers with EGTA does not alter the ability of sarcoplasmic reticulum to sequester and release calcium.¹⁸⁾ We cannot affirm that the EGTA treatment has exactly the same skinning effects on membranes of aortic strips from both SHR and WKY strains. Nevertheless if the SR function is studied with isotopic techniques the chemical skinning is necessary and this uncertainty will not be solved no matter what agent is used to skin the plasma membranes.

The extrapolated values of Ca content in the intracellular compartment were slightly higher in SHR than in WKY, a difference that became significant due to the greater unstimulated rate of ^{45}Ca efflux exhibited by the normotensive group. These results could be in agreement with those reporting that caudal arteries treated with 2 mM EGTA for 30 minutes empty their intracellular Ca stores to a lower degree in SHR than in WKY rats as was estimated by their contractile response to norepinephrine stimulation after the exposure to EGTA.^{1,8)} At 20 minutes of washing, the average Ca content in the intracellular compartment of aortic strips was $163 \pm 7\ \text{nmol/g}^{-1}\ \text{wt}$ for WKY and 217 ± 7 for SHR. This Ca pool releases Ca upon stimulation with micromolar free Ca^{2+} and it is also sensitive to Ca channel blockers of sarcoplasmic reticulum. Thus, a

calculation of its Ca concentration can be made on the basis of a 5% reticular volume in aortic smooth muscle cells¹⁹⁾ and a 35% fraction of smooth muscle cells in the aortic wall.²⁰⁾ The calculated concentrations are 9.3 mM in WKY and 12.5 mM in SHR. The larger concentration in SHR could be apparent if this strain has a larger SR volume compared to WKY. The differences in Ca content could not be attributed to strain variations in smooth muscle content of the aortic wall. Morphometric studies have shown that the fraction of total aortic medial volume occupied by smooth muscle is the same in SHR and WKY rats at the age of those used in the present study.²¹⁾

Several reports showed that the contractile response of vessels is greater in SHR than in WKY rats.^{1,8,11)} In one of these, the mesenteric resistance vessels from SHRs developed more contraction upon epinephrine stimulation than those of WKY rats, but a greater concomitant efflux of Ca could not be demonstrated in the hypertensive strain.¹⁾ The authors suggested that the extrusion of Ca through the ATP-driven pump could be depressed in SHRs⁶⁾ thus masking the efflux of Ca from intracellular stores. Our results show that a greater intracellular pool of Ca can release more Ca upon stimulation with submicromolar and micromolar concentrations of free Ca^{2+} in SHRs. Thus a stimulated contractile response might be expected on this basis in vessels of genetically hypertensive rats. Results on Ca activity measured with fura-2 have suggested an enhanced and prolonged SR calcium release in smooth muscle cells of SHRs.²²⁾

Differences between the amount of Ca released with 0.03 μM free Ca^{2+} (no significant changes in rate coefficient) and the maximal release obtained with higher free Ca^{2+} concentrations permit calculation of the increases in Ca^{2+} in the extracellular space. The values were 2.5 nmol.g⁻¹ wt.min⁻¹ in WKY and 4.2 in SHR. These values might be overestimated if part of the ⁴⁵Ca efflux is ⁴⁰Ca-⁴⁵Ca exchange. We cannot conclude from our present results if the partial blockage of Ca^{2+} -induced ⁴⁵Ca efflux is due to insufficient concentrations of the channel blockers for fully deactivating the SR-Ca channel or to ⁴⁰Ca-⁴⁵Ca exchange.

As shown in Table III the release of Ca is higher in SHR than in WKY when the stimulation affected a compartment with greater Ca content and the rate of Ca efflux was similar in both strains (Figure 3). In spite of the fact that we did not measure the contractile response of our preparations, the present results suggest that contractions elicited by agonists inducing SR Ca release in SHRs will depend primarily on the amount of Ca stored in SR. Results supporting that vascular reactivity is higher in SHR than in WKY were based upon experimental protocols that included pretreatment of vessels with 2 mM of EGTA for 20–30 minutes,^{1,8,11)} and those describing the opposite used 0.1 mM EGTA¹⁰⁾ or did not treat the vessels with EGTA.^{8,9)} One of these studies⁸⁾ showed that after the EGTA treatment, contractions induced by norepinephrine increased with the

time of reexposure to Ca-containing solutions and were always higher in SHR than in WKY caudal arteries. Possibly the treatment with 2 mM of EGTA allow an increased influx of Ca^{2+} through the sarcolemma during the exposure to Ca and more Ca was stored in SR of vascular smooth muscle cells from SHRs.

In summary, the Ca^{2+} -induced Ca^{2+} release mechanism works at micromolar concentrations of free Ca^{2+} in EGTA-treated aortic strips of SHR and WKY rats with the hypertensive strain having a potentially greater content of Ca in the SR of its aortic smooth muscle cells.

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