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COMPARISON OF CALCIUM IMPORT AS A FUNCTION OF CONTRACTION IN THE AORTIC SMOOTH MUSCLE OF SPRAGUE-DAWLEY, WISTAR KYOTO AND SPONTANEOUSLY HYPERTENSIVE RATS

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1. ABSTRACT

Genetic variations of far-reaching consequences have been established between spontaneously hypertensive rats (SHR) and their controls, Wistar Kyoto rats (WKY). The SHR strain is the most widely used model for the study of genetic hypertension. Calcium homeostasis in the vascular smooth muscle (VSM) is controlled by calcium channels and calcium pumps located in both VSM and the overlying endothelial cells that line the large blood vessels and the heart. Hypertension adversely affects calcium homeostasis. Investigations on the import of calcium from extracellular spaces with $alpha_1$-adrenergic stimulation as a function of contractility of VSM cells in SHR and WKY were made and compared with the contractility observed in VSM cells of Sprague-Dawley (CD) rats. Experiments were performed on rings from thoracic aortas of three strains with endothelial lining intact or removed to discern the paracrine control of endothelium on contractility in response to calcium import. The internal stores of Ca^{2+} were depleted by repeated alpha 1-adrenergic stimulation with phenylephrine (PE) and refilling of these stores was prevented by cyclopiazonic acid (CPA) and/or thapsigargin (TG), two known inhibitors of Ca^{2+} ATPase, the enzyme that drives sarcoplasmic calcium pumps. The two components of tonic muscular contraction, T I and T II, which are known to be due to the flow of Ca^{2+} from the extracellular gradient controlled via the poly-phosphoinositide cascade and nifedipine sensitive Ca^{2+} channels were found to be variable among these strains. Implications of these variations are discussed in this report.

2. INTRODUCTION

Spontaneously Hypertensive rats (SHR) and Wistar Kyoto (WKY) Control rats are the most widely used animal models for studies on essential hypertension in humans. Both SHR and WKY rats were established from the normotensive Wistar strain (1). However, due to inbreeding over the years, the SHR and WKY have drifted apart genetically. This has been shown in studies on immunological and biological functions (2), fragment length polymorphism analysis (3,4), DNA fingerprinting (5) and polyploidization of vascular smooth muscle (6). In addition, evidence has been presented that the WKY strain from some sources has been out-bred and shows heterogeneity, but the SHR strain from all different sources shows homogeneity (2). The incidence of hypertension is almost 100 percent in all SHR lines indicating that most of the genes responsible for hypertension have become fixed (1). The number of genes responsible for the hypertensive phenotype has been suggested to be quite small (1). It is more likely that the genetic variations between colonies of SHRs will lead to differences in traits that are not concerned with the development of hypertension. Based on the genetic analysis of RTI^k haplotype of the major histocommpatibility complex (MHC), it has been suggested that SHR and WKY have a similar genetic backgrounds (7). Still, it remains to be established that SHR and WKY are cogenic for hypertension-causing genes.

Hypertension is known to alter aortic contractility because of its accompanying effects on various cellular mechanisms, mostly due to insensitivity of the contractile machinery to the intracellular free calcium ion concentration $[Ca^{2+}]_i$ which provides the central signal for muscular contraction (8,9,10,11,12,13). Defects in the permeability of plasma membrane of VSM cells have been proposed as the central cause for excess $[Ca^{2+}]_i$ in hypertensive VSM cells (14,12). Altered calcium homeostasis in SHR as compared to WKY has been suggested to be a contributing factor for genetic hypertension (15). Thus, it is important to analyze the calcium conductance to and from intracellular stores and the extracellular gradient.

When VSM cells are excited, internal stores of calcium are the first to be utilized. Under continuous stimulation, additional calcium is imported from the extracellular space. The contraction in response to adrenergic stimulation in the absence of extracellular calcium is due to release of Ca^{2+} from internal stores and is termed phasic contraction. Whereas, the contraction in response to extracellular calcium is termed tonic contraction (16). When muscle relaxes, calcium is resequestered into intracellular stores called caveolae by an ATP driven calcium pump of the sarcoplasmic reticulum (SR) in VSM(11). Another type of pump is employed for pushing calcium out against the gradient to the extracellular spaces (8,17,18) . The SR Ca^{2+} pump can be blocked effectively by calcium ATPase blockers such as cycolpiazonic acid and thapsigargin (19,20,21,22,23,24).

DNA analysis and histocompatibiltiy studies of haplotypes have shown that SHR and WKY rats have different genetic profiles which implies that other cell membrane proteins such as ion channels might also show variability among the two strains of rats. Additionally, Ca^{2+} channel expression defects have been established in SHR neonates without assignment of any causal function to this defect in the development of high blood pressure (15). To elucidate any biophysical differences at that level, this study compares the import of calcium ion through cell membrane channels from extracellular spaces as a function of contraction in response to alpha-adrenergic stimulation of smooth muscles in aortic rings from Sprague Dawley, Wistar Kyoto and spontaneously hypertensive rats (SHR).

3. MATERIALS AND METHODS

Male rats of the SHR, WKY and CD strains were purchased from Charles River, Wilmington, Massachusetts, USA. Upon arrival, the animals were acclimatized for 24 hours. They were kept in ventilated animals racks (Edstrom Industries). Drinking water was provided through a continuous watering system (Edstrom Industries). Purina rat food together with tap water was provided *ad libitum*. The day was divided into 12 hours of darkness and 12 hours of light periods. Indirect systolic blood pressure in the tail artery was measured the day animals were received and one day prior to sacrifice. The average body weight in grams of CD, WKY and SHR animals the day of sacrifice was 327.69 ± 27.48, 275.89 ± 31.72 and 281.85 ± 18.19 respectively. The sacrifice was carried out by a sharp blow to the head followed by cervical dislocation. The aortic rings were prepared and isometric contractions were measured as described earlier (26,27,28,29,30). Isometric contractions were recorded using Gould GM3 microdisplacement myographic transducer coupled to an eight channel physiographic recorder (Gould model RS 3800).

In order to remove the paracrine effects of the endothelium, aortic contraction in each strain was compared for rings with intact endothelium to rings denuded of their endothelium (DND). Prior to testing for contraction in response to calcium import, rings were tested for their viability by invoking contractile response to cumulative dose (8-80 mM) of potassium chloride (KCl). A second purpose of this stimulus is the liberation of norepinephrine from adrenergic nerve terminals in isolated vascular segments or rings (31). Removal of endothelium was accomplished by rolling the clean ring three times on tissue paper soaked in Krebs solution with an L-shaped lightly filed steel rod (0.71 mm diameter) and gently pipetting the Krebs solution with the help of a Pasteur pipette through the lumen of the aortic ring. The absence of relaxation in response to acetylcholine was considered confirmation of denuded endothelium.

3.1. Contraction in Response to Ca^{2+} Import from Extracellular Gradient

To evaluate contraction in response to calcium import from extracellular spaces, the rings were depleted of internal calcium stores by repeated alpha-adrenoceptor stimulation with 10^{-6} M phenylephrine (PE) in calcium free Krebs solution. Rings were allowed to contract with each stimulation until contractions plateaued or for at least a 7 minute period after the injection of PE. After depletion was achieved, rings were maintained in two concentrations (5 µM and 10µM) of cyclopiazonic acid (CPA) and one concentration (10 µM) of thapsigargin (TG) to block the SR calcium pumps. To test the blockade, Ca^{2+} was restored in the Krebs solution in presence of CPA and TG for a brief period before placing the rings in Ca^{2+} free Krebs and stimulation with 10^{-6} M PE. The contractile response in Ca^{2+} free Krebs with blockers in place was given the designation of residual contraction. To measure the contraction in response to calcium import from the extracellular gradient, the rings were incubated in Ca^{2+} free Krebs and Ca^{2+} pump blockers for 30 minutes after the residual contraction. Following this equilibration period, rings were stimulated with 10^{-6} M PE in Ca^{2+} free Krebs in the presence of Ca^{2+} pump blockers CPA and TG. Two minutes after the injection of 10^{-6} M PE calcium was restored in the Krebs solution bathing the tissues by injecting 200 µl of 0.25 M/l $CaCl_2$ solution. Under these conditions two distinct components of the tonic contraction were observed; tonic I and a superimposed tonic II. Initially, the muscle contracts continuously for 2 to 3 minutes followed by a short plateau. A second, superimposed contraction appears which has been shown to be susceptible to blockage by nifedipine (16).

The procedures for animal maintenance, blood pressure recordings and vivisections to prepare aortic rings were reviewed and approved by the Institutional Animal Care and Use Committee of Bethune-Cookman College.

3.2. Solutions and chemicals

Composition of the Krebs solution was 118 mM NaCl, 4.7 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM MgCl, 12.5 mM $NaHCO_3$, 11.1 mM Dextrose and 0.01 mM NaEDTA. The chelator ethyleneglycol bis (b-aminoethyl ether) N,N,N',N'-tetra acetic acid (EGTA) in 0.5 mM concentration was used in Ca^{2+} free Krebs solution. The Ca^{2+} free Krebs solution was used to remove the extracellular gradient of Ca^{2+} and to wash out the $[Ca^{2+}]_i$ from the cytosol following stimulation of the VSM with the alpha-adrenoceptor agonist PE.

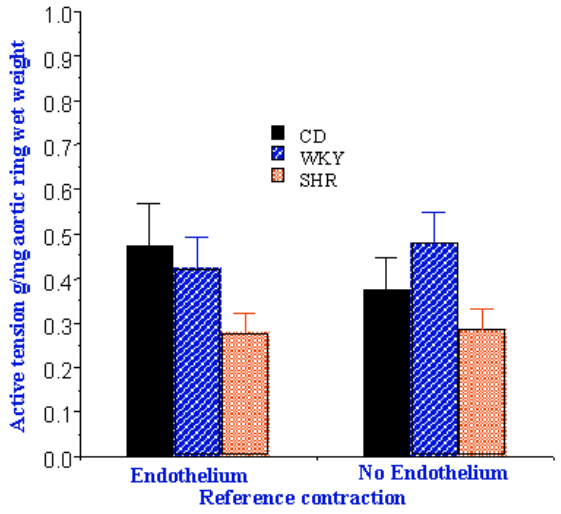

Figure 1. A comparison of sub-maximal reference contractions elicited in response to preferential alpha1-agonist phenylephrine (PE) by aortic rings with and without monolayer of endothelial cells from Sprague-Dawley (CD), Wistar Kyoto (WKY) and Spontaneously Hypertensive Rats (SHR). The data represents mean and standard error of means (SEM) for contractility from a minimum of 12 rats of each strain. The hats on bars represent ± SEM.

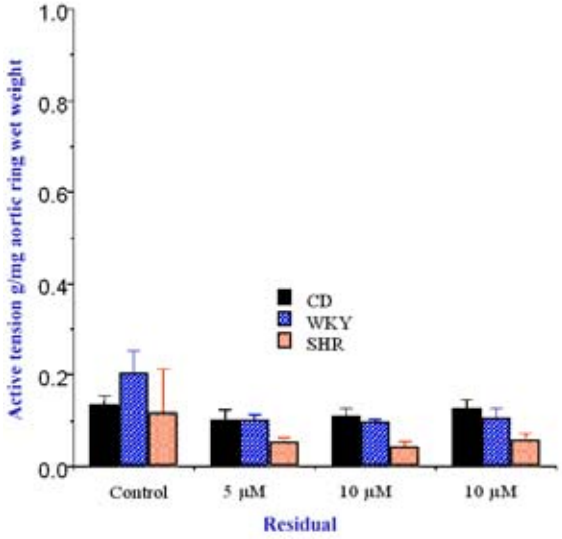

Figure 2. A comparison of residual contraction produced by aortic rings of CDs, WKYs and SHRs with intact monolayer of endothelium and bathing in Ca^{2+} free Krebs solution in the presence of CPA and TG. Control rings did not have any SR calcium pump blockers in their environment. The contractions were produced when 10^{-6}M PE was injected into the muscle baths. The data represents mean and standard error of means (SEM) for contractility from a minimum of 6 rings from each strain of rats. The hats on bars represent ± SEM.

CPA stock solution (2 x 10^{-2} M) was made by dissolving 0.5 mg of CPA in 743 µl of Dimethyl Sulfoxide (DMSO) and 1.5 x 10^{-2} M TG stock solution was prepared by dissolving 9.77 mg of TG in 1 ml of DMSO. Krebs solution and all other solution were prepared using double distilled deionized water.

All chemicals for Krebs solution, cyclopiazonic acid, thapsigargin and the alpha-agonist phenylephrine hydrochloride were purchased from Sigma Chemical Company, USA.

3.3. Statistical Analysis

The Analysis of Variance (ANOVA) was employed to determine differences in import of calcium among the rat strains (CD, WKY and SHR). In addition, the ANOVA was used to ascertain the effects of varying concentrations of SR calcium pump blockers (cyclopiazonic acid at 5 and 10 µM and thapsigargin at 10 µM concentrations) on tonic -I and tonic-II components of aortic ring contractions among the three strains of rats with and without the monolayer of endothelial lining. The values reported are mean ± standard error of means (SEM) from a minimum sample size of 6. Significance levels of probability were tested at $p < 0.05$ and 0.01.

4. RESULTS AND DISCUSSION

The endothelium is known to regulate vascular smooth muscle tone through the production of vasoconstrictors, endothelins, and the vasorelaxents, nitric oxide (NO) and prostacyclins, namely PGI_2. Intracellular free calcium plays the role of central second messenger in endothelium for its paracrine function (20,32,33,34,35,36). We studied the sub-maximal contraction of VSM in response to 10^{-6} M phenylephrine (PE), a preferential $alpha_1$-agonist in all three strains and designated this response as reference contraction. Sub-maximal reference contraction in the presence of intact monolayer of endothelium was largest in CD rings and smallest in rings from SHR animals as shown in figure 1. The overall results of experiments in the presence of intact monolayer of endothelium are summarized in table 1. When the endothelium was removed from rings, the reference contraction in WKY produced a large increase, the contraction amplitude in SHR was unchanged and rings from CD rats produced a decrease of 25.67% in sub-maximal reference contraction. The latter results together with residual and tonic contractions from denuded rings are presented in table 2. This variability in sub-maximal contraction pattern confirms the reported endothelial dysfunction associated with hypertension pathology.

4.1. Comparison of Calcium Import In VSM of Sprague Dawley, WKY and SHR Aortic Rings With Intact Endothelium

The results from experiments to assess calcium import from the extracellular gradient in the presence of an intact monolayer of endothelium are presented in table 1. When 10^{-6} M PE is introduced in the tissue bath there was a small contraction which is termed residual contraction. A comparison of residual contraction in the presence of intact monolayer of endothelium (figure 2) shows that, in the absence of any Ca^{2+}-ATPase inhibitors of SR calcium-pumps, WKY rings produced significantly higher responses

Table 1. Comparison of contraction components in Aortic Ring Smooth Muscle with intact Endothelial Monolayer of the lumen

SR Pump Blocker	Contraction Type	Sprague Dawley Rats	Wistar Kyoto Control Rats	Spontaneously Hypertensive Rats
No Blocker	Reference	0.470 ± 0.100	0.422 ± 0.068	0.280 ± 0.042
No Blocker	Residual	0.133 ± 0.021	0.205 ± 0.046	0.118 ± 0.096
No Blocker	Tonic I	0.735 ± 0.076	0.598 ± 0.077	0.592 ± 0.072
No Blocker	Tonic II	1.230 ± 0.137	1.093 ± 0.105	1.033 ± 0.042
5 µM CPA	Residual	0.101 ± 0.021	0.102 ± 0.013	0.054 ± 0.011
5 µM CPA	Tonic I	0.797 ± 0.097	0.497 ± 0.048	0.693 ± 0.067
5 µM CPA	Tonic II	1.523 ± 0.158	1.124 ± 0.082	1.247 ± 0.071
10 µ M CPA	Residual	0.111 ± 0.014	0.099 ± 0.004	0.044 ± 0.010
10 µ M CPA	Tonic I	1.045 ± 0.101	0.455 ± 0.065	0.657 ± 0.023
10 µ M CPA	Tonic II	1.904 ± 0.198	0.998 ± 0.083	1.178 ± 0.062
10 µM TG	Residual	0.128 ± 0.017	0.107 ± 0.021	0.061 ± 0.009
10 µM TG	Tonic I	0.834 ± 0.062	0.442 ± 0.029	0.616 ± 0.063
10 µM TG	Tonic II	1.500 ± 0.124	1.047 ± 0.081	1.243 ± 0.129

The values are means ± standard error of means for a minimum sample size of 6

Table 2. Comparison of contraction components in Aortic Ring Smooth Muscle without the Endothelial Monolayer of the lumen

SR Pump Blocker	Contraction Type	Sprague Dawley Rats	Wistar Kyoto Control Rats	Spontaneously Hypertensive Rats
No Blocker	Reference	0.374 ± 0.073	0.478 ± 0.069	0.287 ± 0.042
No Blocker	Residual	0.125 ± 0.016	0.155 ± 0.017	0.145 ± 0.043
No Blocker	Tonic I	0.809 ± 0.095	0.748 ± 0.043	0.621 ± 0.096
No Blocker	Tonic II	1.287 ± 0.170	1.200 ± 0.059	1.033 ± 0.042
5 µ M CPA	Residual	0.079 ± 0.018	0.102 ± 0.013	0.054 ± 0.011
5 µ M CPA	Tonic I	0.701 ± 0.067	0.497 ± 0.048	0.693 ± 0.067
5 µ M CPA	Tonic II	1.014 ± 0.175	1.124 ± 0.082	1.247 ± 0.071
10 µ M CPA	Residual	0.134 ± 0.010	0.099 ± 0.004	0.044 ± 0.010
10 µ M CPA	Tonic I	0.864 ± 0.069	0.455 ± 0.065	0.657 ± 0.023
10 µ M CPA	Tonic II	1.419 ± 0.090	0.998 ± 0.083	1.178 ± 0.062
10 µM TG	Residual	0.093 ± 0.019	0.107 ± 0.021	0.061 ± 0.009
10 µM TG	Tonic I	0.640 ± 0.077	0.442 ± 0.029	0.616 ± 0.063
10 µM TG	Tonic II	1.036 ± 0.099	1.047 ± 0.081	1.243 ± 0.129

The values are means ± standard error of means for a minimum sample size of 6.

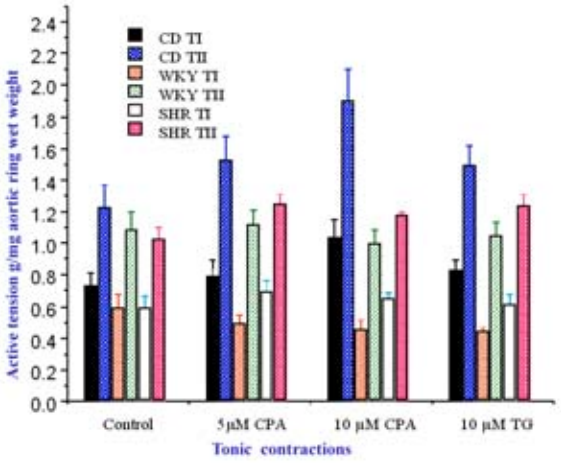

Figure 3. A comparison of contractions in response to import of Ca^{2+} from the extracellular spaces. The initial component tonic I (TI) and a delayed component tonic II (TII) were elicited from aortic rings with intact monolayer of endothelium. The stimulus for calcium import was provided by $alpha_1$-preferential agonist PE at a concentration of 10^{-6} M to rings from CD, WKY and SHR animals. Sarcoplasmic calcium pump blockers cyclopiazonic acid CPA and thapsigargin (TG). CPA was used at concentrations of 5 µM and 10 µM where as only 10 µM dose was used for TG. The data represents mean and standard error of means (SEM) for active tension from a minimum of 6 rings from each strain of rats. The hats on bars represent ± SEM.

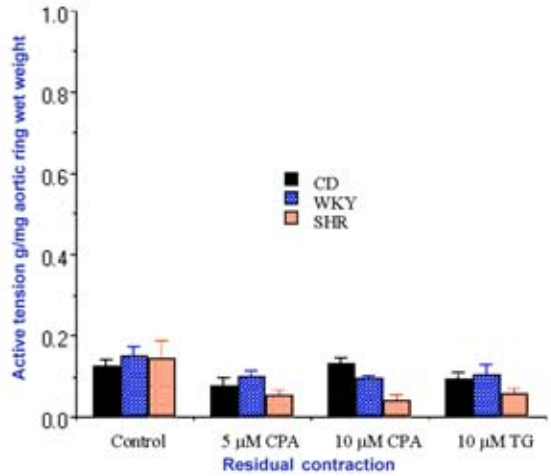

Figure 4. A comparison of residual contraction produced by aortic rings of CDs, WKYs and SHRs without the monolayer of endothelium. The rings were bathing in Ca^{2+} free Krebs solution in the presence of CPA and TG. The control rings did not have any SR calcium pump blockers in their environment. The contractions were produced in when 10^{-6}M PE was injected into the muscle baths. The data represents mean and standard error of means (SEM) for contractility from a minimum of 6 rings from each strain of rats. The hats on bars represent ± SEM.

than either the CD or the SHR aortic rings. Two concentrations of cyclopiazonic acid (CPA), 5 µM and 10 µM, had comparable inhibitory effects, whereas, 10 µM thapsigargin (TG) was slightly less effective as rings from all three strains produced higher responses as compared to rings treated with CPA.

Restoration of the extracellular gradient of 2.5 mM calcium while the SR Ca^{2+} pump blockers are present in the Krebs solution bathing the rings caused the rings to contract due to an increase in $[Ca^{2+}]_i$ from calcium import from the extracellular gradient under the alpha-adrenergic stimulation provided by a sub-maximal 10^{-6} M PE dose. This contraction can be distinguished into two components: an immediate response lasting for 2 to 3 minutes termed tonic I contraction, followed by a second contraction termed tonic II. As reported in the literature (16) the tonic I contraction is due to involvement of the polyphosphoinositide cascade and tonic II is dependent on calcium influx through the nifedipine sensitive Ca^{2+} channels. We did not measure the calcium uptake rates during these components of tonic contraction. However, from the amplitude of contractions, an estimate of the relative amount of calcium can be deduced. CPA concentration of 5 µM and TG concentration of 10 µM were equally effective in increasing the [Ca2+]i in response to alpha1-adrenergic stimulation by 10-6 M concentration of PE in all three strains (figure 3), but CPA concentration of 10 µM produced significantly higher tonic I as well tonic II contractions in aortic rings of CD rats, indicating a more effective blockade of SR calcium pump by a higher concentration of CPA in this strain as compared to SHRs and WKYs. Another interesting fact noticed was the relative amplitudes of tonic I and tonic II contractions. It appears, CPA and TG possibly suppress the polyphosphoinositide cascade-mediated tonic I contraction, but enhance the tonic II contraction which is caused by the import of calcium through the nifedipine sensitive Ca2+ channels.

4.2. Comparison of Calcium Import In VSM of Aortic Rings Without Endothelium Among Sprague Dawley, WKY and SHR

Since the endothelium has been shown to exercise a paracrine effect on the underlying vascular and cardiac muscle through substances such as endothelin, nitric oxide and prostanoids (8,14,20,32,33,34, 35,37), we denuded the monolayer of endothelial cells to investigate contractility in aortic rings due to extracellular calcium import directly into VSM cells. Overall results of these experiments with denuded (DND) rings are summarized in table 2. Residual contraction in DND rings (figure 4) has a different pattern than in rings with intact endothelium (figure 2). Removal of endothelium significantly reduced the amplitude of residual contraction in the absence of SR calcium pump blockers (CPA and TG) in rings from WKY and enhanced the amplitude of contraction in SHR rings. The use of CPA and TG in the denuded rings did not alter significantly the profiles of contractions from those obtained from rings with intact endothelium. A comparison of tonic contractions in denuded rings (figure 5) to the rings with intact endothelium (figure 3) indicates a significant decrease in amplitudes at both concentrations of CPA and at the single concentration of TG. These results affirm the observations of others that CPA and TG

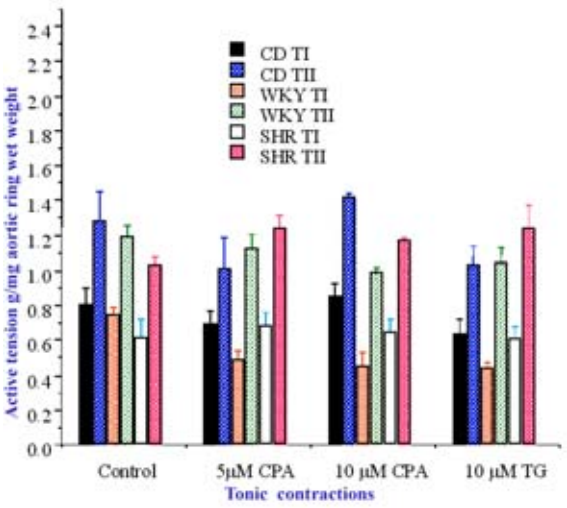

Figure 5. A comparison of contractions in response to direct import of Ca^{2+} into the vascular smooth muscle of thoracic aortas from the extracellular spaces. The initial component TI and a delayed component TII were elicited from aortic rings with their monolayer of endothelium removed. The stimulus for calcium import was provided by 10^{-6} alpha 1-preferential agonist PE at a concentration of 10 M to rings from CD, WKY and SHR animals. Sarcoplasmic calcium pump blockers cyclopiazonic acid CPA and thapsigargin (TG). CPA was used at concentrations of 5 µM and 10 µM where as only 10 µM dose was used for TG. The data represents mean and standard error of means (SEM) for active tension from a minimum of 6 rings from each strain of rats. The hats on bars represent ± SEM.

modulate contractility of $_{2+}$underlying smooth muscle by altering the levels of $[Ca^{2+}]_i$ in endothelial cells and thus the function of the endothelium (20,32,33,23,34). A comparison of tonic contractions in the presence of 10 µM CPA showed that removal of endothelium did not alter the amplitude of tonic II contraction in WKY and SHR rats as much as it did in the CD rats. From these results, it may be inferred that the nifedipine-sensitive Ca^{2+} channels of endothelial cells as well as VSM cells of CD rats are different than in both the WKY and the SHR. In fact, it appears that these channels behave more alike in hypertension prone SHRs and their control WKYs.

5. SIGNIFICANCE

Despite recent reports of genetic heterogeneity, SHR and WKY rats continue to be the most widely used animal model for the study of genetic hypertension. In addition, patterns of hypertensive pathology and cardiovascular structure and function in SHR are quite similar to humans. The hypertensive phenotype in SHR is polygenic (12) and very few of these genes have been located and characterized (7). The limited genetic analysis has provided contradictory information. For example, similar genetic backgrounds for SHR and WKY have been proposed for RTI^{K} haplotype of the major histocompatibility complex, but the analysis of rat major histocompatibility complex for heat-shock protein 70 (hsp 70) locus showed no linkage, and, by implication, no linkage for other genes located within RT1 complex and blood pressure between SHR and WKY rats (39). On the other hand, genetic determination of calcium handling has been reported in experiments with Ren 2-transgenic rats (12). The expression of transgene was found to be high in vasculature and this expression is accompanied by an increase in local formation of angiotensin II which, in turn, indicates alterations in beta-adrenergic neuroeffector mechanisms, Ca^{2+}-handling and alpha-adrenergic signal transduction by association. In the light of this information, we have observed the contractile behavior of aortic rings in response to Ca^{2+} import from the extracellular space in Sprague-Dawley, Wistar Kyoto and spontaneously hypertensive rats.

The fact that removal of endothelium did not affect the reference contraction in SHR aortic rings confirms that, in hypertension, the endothelium becomes dysfunctional. However, when we consider the behavior of WKY control VSM, the absence of endothelium enhances the contractility significantly, whereas in CD, the effect of endothelial removal is the opposite. We found that endothelial control of VSM contractility is variable in these three strains. The cause of this variability could be differences in the Ca^{2+} handling mechanisms and consequent expression of paracrine products such as endothelins, NO and prostacyclins which act as vasoreactive agents.

These studies report variations in the tonic contractions in response to import of Ca^{2+} due to alpha-adrenoceptor activation among the three strains. Pursuing the classification of tonic I contraction as a polyphosphoinositide cascade-controlled contraction and tonic II contraction as a result of calcium import through the nifedipine-sensitive calcium channels as proposed by Nishimura *et al* (16), it is purposed that when SR Ca^{2+}-ATPase is not blocked, the removal of endothelium significantly reduces the tonic II component. The blockade of sarcoplasmic reticulum Ca^{2+} pump again produces variable effects on the tonic II component of contraction. In the presence of CPA or TG, the VSM from CD rats produced smaller contractions in denuded aortic rings than in non denuded rings. Removal of endothelium and blockage of SR Ca^{2+}-ATPase by CPA and TG did not affect the magnitude of tonic II component in the VSM of WKY and SHR aortic rings. However, the magnitude of tonic II contraction component both with and without endothelium was significantly higher than the tonic II contraction in SHR indicating that nifedipine-sensitive Ca^{2+} channels are more efficient in WKY rats than in the SHR regardless of the paracrine activities of endothelium. Whether this is due to genetic causes or a consequence of hypertensive pathology needs to be elucidated.

6. ACKNOWLEDGMENT

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