Intracellular Vascular Muscle Ca\textsuperscript{2+} Modulation in Genetic Hypertension

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Distribution of intracellular free calcium concentration (Ca\textsuperscript{2+}) was compared in spontaneously hypertensive rat (SHR) and Wistar-Kyoto (WKY) rat isolated vascular muscle cells at rest and during stimulation by K\textsuperscript{+} with Ca\textsuperscript{2+} agonist or antagonist. Ca\textsuperscript{2+} activity was quantitated at each point within vascular muscle cells loaded with fura-2 at fluorescence excitation wavelengths of 340, 360, and 380 nm, and fluorescence emission at 510 nm (all filters were ±5 nm) quantitated by a digital photon-counting camera. Measurements of fluorescence intensity ratio in central and subsarcolemmal areas showed that calcium release, in response to 30 or 100 mM K\textsuperscript{+} with Ca\textsuperscript{2+} agonist or during spontaneous contractions, was principally from sarcoplasmic reticulum. Addition of the Ca\textsuperscript{2+} agonist Sdz 202-791, S (+) stereoisomer (SdzS), caused a dose-dependent increase of Ca\textsuperscript{2+} in both SHR and WKY rats. Intracellular calcium release sites were defined by “hot spots” of high fluorescence intensity ratio in both central and peripheral regions of the sarcoplasm. The size and intensity of hot spots increased, and there was an initial transient activation of subsarcolemmal calcium pools in response to high K\textsuperscript{+} with 1 μM Ca\textsuperscript{2+} agonist. In contrast, treatment of the cells with the R (-) stereoisomer of Sdz 202-791 (SdzR), a Ca\textsuperscript{2+} antagonist, prevented the increase in Ca\textsuperscript{2+} and the increase in hot spot size by either K\textsuperscript{+} alone or with agonist. Antagonist decreased central core Ca\textsuperscript{2+} release and fragmented the subsarcolemmal hot spots. The difference between the effect of SdzS on cells of SHR versus WKY rats was a greater enhancement by SdzS of intracellular Ca\textsuperscript{2+} in SHR than WKY rats, especially at the peripheral rim. Furthermore, peripheral Ca\textsuperscript{2+} activity reached higher levels in SHR than were found in WKY rats, probably indicating a submembrane Ca\textsuperscript{2+} regulation deficiency. Antagonist (SdzR) reduced total cell intracellular Ca\textsuperscript{2+} to control levels in both SHR and WKY rats, which were not different. These findings suggest fundamental differences in vascular muscle cells of SHR and WKY rats, which were evident even in single cells isolated from veins of neonatal animals, implicating Ca\textsuperscript{2+} channels and modulation of release and uptake that would be important for both short-term and long-term regulation of Ca\textsuperscript{2+} activity. (Hypertension 1989;14:145-151)

The trigger step that initiates the increase in intracellular free calcium concentration (Ca\textsuperscript{2+}) is an extremely important component of excitation in vascular muscles.\textsuperscript{1-3} Electron probe studies have suggested that the processes and pathways of calcium entry, release, and reuptake in vascular muscle differ markedly among blood vessels.\textsuperscript{1,4} Calcium release from sarcoplasmic reticulum (SR), where it is stored in high concentrations, appears to be the major source for activation of contraction and provides the possibility of heterogeneously activating contraction within a single cell.\textsuperscript{1,4,5} Released calcium is avidly taken up by the SR, and there is evidence for a large number of high affinity calcium pumps in both the SR and the sarcolemma.\textsuperscript{6,7} These calcium control mechanisms are functionally sufficient to establish concentration gradients for free Ca\textsuperscript{2+} (precisely termed Ca\textsuperscript{2+} activity) within a single cell. Thus, physical barriers are not necessary for the existence of different Ca\textsuperscript{2+} activities within a cell, even though enzymes that produce regions with altered Ca\textsuperscript{2+} activity are primarily organized by membranes, such as SR.

Several lines of evidence point to altered calcium handling associated with the development of hypertension.\textsuperscript{8-10} Mulvany et al\textsuperscript{11} and Whall et al\textsuperscript{12} reported oscillations of intracellular Ca\textsuperscript{2+} on return to Ca\textsuperscript{2+} from Ca\textsuperscript{2+}-deficient solution to be an intrinsic defect in vascular muscle of resistance vessels, which could possibly contribute to the development...
of hypertension in spontaneously hypertensive rats (SHR). Evidence for an inverse correlation of plasma Ca2+ and blood pressure has been reported in humans13 and animals.14 There is evidence for increased Ca2+ in erythrocytes15 and platelets of hypertensive humans.16 Recent data from our laboratory suggest that a differential action of nitren- dipine and related dihydropyridines might be found in SHR versus Wistar-Kyoto (WKY) rat vascular muscle because of Ca2+ channel differences.17

Our attention has been drawn to the stereoisomers of the dihydropyridine, Sdz 202–791, which have opposite effects on calcium channels.18 The S (dextrorotatory +) stereoisomer (SdzS) is a calcium agonist and the R (levorotary –) stereoisomer (SdzR) is an antagonist, at least at concentrations from 10 nM to 1 μM. Because our electrophysiological experiments suggested that dihydropyridines have an action on SR as well as on surface membrane,2,19 we investigated the actions of the Sdz 202–791 racemates on subcellular calcium distribution in vascular muscle cells of SHR and WKY rats. Our experiments, using extremely high sensitivity and resolution in living vascular muscle cells, continue to implicate altered regulation of intracellular calcium as an important vascular muscle change in hypertension.

Materials and Methods

Cell Isolations From Azygous Veins

Primary cultures of vascular muscle cells were prepared from azygous veins of 3-day-old SHR and genetically matched WKY rats, as described in detail elsewhere.20 The cells were dissociated and plated at low density (50–100,000 cells) onto polylysine-coated or uncoated glass coverslips.

Calcium Quantitation From Isolated Single Cells

Two days later, the coverslips were placed in a laminar flow chamber,21 and a single spontaneously contracting vascular muscle cell was identified and observed at ×750 magnification on a Leitz Diavert microscope. To load cells with fura-2, 20 μl of fura-2 acetoxymethylester stock solution (1 μM fura 2-AM in dimethyl sulfoxide [DMSO] containing 0.05% pluronic 127) was added to the 300-μl chamber. After 5 minutes, extraneous fura-2 was washed away with ionic solution for mammals (ISM) consisting of (mM): NaCl 130, NaHCO3 16, KCl 4.7, CaCl2 1.8, MgCl2 0.4, MgSO4 0.4, Na2HPO4 0.5, and HEPES 17, with dextrose 5.5 and CaNa2EDTA 0.03. This comparatively short loading procedure was repeated if the cells failed to take up sufficient fura-2 (only two cells of 150). Temperature during the entire procedure was kept constant at 37° C.

Fluorescence intensities were detected and digitized at 340, 360, and 380 nm excitation and 510 nm emission by a microchannel plate, photon-counting camera (model VIM, Photonic Microscopy, Inc., Oak Brook, Illinois), using 10 nm bandwidth filters from Corion and Oriel. This ultrahigh-sensitivity camera permitted absolute quantitation with extremely low light levels that produce little or no fading.22 Daily variability of the light source and detector was measured and corrected (see equations below) using standardized fluorescence calibration beads (GTE Sylvania, Towanda, Pennsylvania) cemented in the chamber at ×750 magnification; resolution of the cell image was 0.3 μm. Continuous photon-counting recordings were made with a Sony VO5800 video-cassette recorder, stored on a Hewlett-Packard (Cupertino, California) Vectra computer, and transferred to an ISI archiving laser disk.

Determination of calcium is based on triple wavelength analysis of the calcium-dependent fluorescence ratio on a pixel/pixel basis. For analysis of intracellular calcium, digital frame memories at standardized time points were interrogated at each pixel for 510 nm fluorescence intensity in images accumulated for either 0.25 or 0.5 seconds, as required for adequate signal-to-noise ratio. The ratio of fluorescence intensity with excitation at 340 nm divided by that at 380 nm was used to maximize sensitivity to changes in calcium. The concentration of fura-2 at every pixel was measured by fluorescence intensity at 360 nm, the calcium-insensitive excitation wavelength. Differences in cell thickness also appeared as differences in intensity at 360 nm, and are thus included in this concentration correction. For the Ca2+ calibration, the intensity for each pixel at 360 nm excitation was measured and used to provide absolute calibration of calculated Ca2+ activity as detailed below. Regions of high or low fura-2 concentration were found to be nearly constant throughout the experiment, in contrast to Ca2+ activity, which was changing with contraction and relaxation. We do not stress the absolute values of Ca2+ activity, only the relative values, as the fura-2 is a Ca2+ chelator very likely to alter Ca2+ activity in the cytoplasm. Statistical analysis was by unpaired t test for comparison of SHR with WKY rats and by paired t test for comparison before and after drugs within rat strains. The p = 0.05 level of significance was defined as the criterion for acceptance of differences.

The nonhomogeneous distribution of calcium necessitated definition of regions of high fluorescence intensity. We therefore separated regions with more than two times the intensity of the (concentration corrected) mean of the entire cell and defined them as “hot spots.” To separate the central and peripheral regions, we separately quantitated the area of the cell rim (within 0.5 μm, which is 3 pixels from the edge of the cell) and the rest of the cell. This arbitrarily defined region at the cell border is a valid measure of part of the peripheral Ca2+, but the central regions contain a contribution of overlying or underlying peripheral regions as well. Without a scanning microscope, it was not possible to separate fluorescence in the Z axis.
Thus, it is more accurate to label the central regions as the central core, thereby acknowledging the peripheral contribution. Despite this complication, the purely peripheral rim contribution to the edge fluorescence is sufficiently unlike the central core fluorescence to reveal the differentiation we have described. For the triple wavelength calculation of Ca$^{2+}$, we have used:

$$\text{Ca}^{2+} = K_d \times \left( xR - 1.36 \right) / \left( 3.48 - xR \right)$$

where $x$ corrects for fura-2 distribution, fluorescence absolute intensity, and camera accumulation times, determined by computer fit as

$$x = y + Zv e^{-W/\tau}$$

and where $K_d$ is the dissociation constant (239 nM in these experiments), $R$ is the ratio of 510 nm fluorescence excited by 340 nm divided (point by point) by that at 380 nm, $F$ is the fluorescence (510 nm) excited by 360 nm light, $x$ is derived as stated, $y$ is a constant derived from best fit (0.92 in these experiments), $Z$ is a constant derived from best fit (2.24 in these experiments), $W$ is a constant derived from best fit (0.0028 in these experiments), $v$ is the factor from the fluorescence standard, and $d$ is the factor for different camera exposure times.

Drugs used in these experiments were $S$ and $R$ stereoisomers of Sdz 202–791, kindly provided by Dr. R.P. Hof of Sandoz, Ltd., Basel, Switzerland. Nonfading fluorescent calibration beads were provided by GTE Sylvania, Towanda, Pennsylvania. Fura-2 was purchased from Molecular Probes Inc., Eugene, Oregon, and chemicals were purchased from Sigma Chemical Company, St. Louis, Missouri, or Fisher Scientific, Pittsburgh, Pennsylvania.

**Results**

In response to stimulation by elevated potassium solutions, increased calcium concentrations were found within vascular muscle cells, as illustrated in Figure 1. On addition of 30 mM potassium with 100 nM Sdz$S$ (agonist), there was an increase in intracellular calcium that first occurred prominently at the periphery (Figure 1B), and then increasing across the central part of the vascular muscle cells (Figure 1C). The central areas released large Ca$^{2+}$ stores, and total Ca$^{2+}$ activity increased at least fourfold, but regions of lower Ca$^{2+}$ persisted (black or violet areas within cell in Figures 1B and 1C). Intracellular Ca$^{2+}$ was nonuniform at rest and during stimulation. Immediately after addition of Sdz$R$ (antagonist), the Ca$^{2+}$ activity fell, with greatest decreases in Ca$^{2+}$ activity in central regions (Figure 1D). Averages of total, peripheral, and central Ca$^{2+}$ regions were all increased, significantly more in SHR than WKY rats, by Sdz$S$ in 30 mM or 100 mM K+, as shown in Figure 2. Separate quantitation of central core and peripheral rim areas revealed a significantly greater peripheral increase in basal levels of Ca$^{2+}$ activity in SHR than WKY rats. There was a significant increase in Ca$^{2+}$ at the peripheral rim in both SHR and WKY rats with 100 nM (smaller) or 1 µM (larger) Sdz$S$ (paired $t$ test), and Ca$^{2+}$ in SHR increased significantly more than in WKY rats in this region (unpaired $t$ test, Figure 2). The increase in Ca$^{2+}$ was dose dependent, with insignificant increases at 30 nM Sdz$S$ under these conditions. On stimulation, the clearly evident increase of Ca$^{2+}$ activity in cells from SHR was noted, and five additional experiments were carried out to increase the N value to 15 and 19 (as shown in Figure 2) to determine if resting Ca$^{2+}$ was greater in the periphery of cells from SHR. Compared with WKY rats, the increased Ca$^{2+}$ activity at the periphery of even the resting cells of SHR was significant ($p<0.05$, unpaired $t$ test).

Further analysis of group data was carried out by computer analysis of hot spots within the cells. By scanning all of each selected image frame, using a criterion of a twofold increase of Ca$^{2+}$ over the whole cell average, and logically connecting uninterrupted areas, the C program detected from three to 24 hot spots in each cell and determined the average size of a hot spot. When small areas (less than 0.3 µm$^2$) were excluded to allow these data to reasonably represent the extent of occurrence of areas of significant Ca$^{2+}$ activity, there were almost always at least five hot spots at rest and a transient increase in hot spots during stimulation. Figure 3 shows that the number of hot spots remained relatively constant throughout the experiments. Sdz$S$ in 100 mM K+ did not increase the number in SHR or WKY rats. There is only a hint of an early (0–5 second) decrease and late (60–120 second) increase in number by Sdz$R$. However, the size of hot spots was significantly increased by 100–1,000 nM Sdz$S$ in 100 mM K+, as shown by Figure 4. Hot spot size increased in cells of both SHR and WKY rats at 10–30 seconds, with a decrease to control size at 60–120 seconds (Figure 4). Furthermore, size of SHR cells increased significantly more than those of WKY rats (10–30 seconds) by unpaired $t$ test. Sdz$R$ blocked the increase in size of hot spots in cells of both SHR and WKY rats, and there was an insignificant but consistent tendency for Sdz$R$ to reduce hot spot size in each experiment.

The most striking difference in SHR and WKY rats was the peripheral Ca$^{2+}$ activity increase during especially the latter stages (after 10 seconds) of stimulation. Calculations of peripheral/central Ca$^{2+}$ ratios were greater in SHR than WKY rats both during stimulation and at rest (Figure 2).

**Discussion**

These experiments show that genetic hypertension differences in the calcium steps in excitation can be identified using racemates of Sdz 202–791, which have opposite (agonist vs. antagonist) effects on vascular muscle. The data continue to suggest, as previously reported, that there is stereoselectivity at the vascular muscle calcium channel as
clearly demonstrated by the enantiomers of this 1,4-dihydropyridine.

Perhaps most significantly, these experiments identify central versus peripheral intracellular calcium sites as intracellular stores differentially affected by SdZR that, along with the surface mem-

brane calcium channels, may explain the vasodilator properties. It is the central–peripheral shift of Ca\(^{2+}\) that appears to dominate regulation of intracellular Ca\(^{2+}\) in the critical region just inside the surface membrane (K. Hermsmeyer and P. Erne, unpublished observations). Furthermore, the comparison of SHR and WKY rats shows that there must be an alteration in the intracellular mechanisms that are sensitive to these agents, which is evident even before blood pressure is significantly increased. The cells studied in our experiments were from azygous veins of 3-day-old rats, in which blood pressure differences would not be expected to play any role. Because sustained Ca\(^{2+}\) channels are inactivated by Ca\(^{2+}\) at the inner edge of the cell membrane in azygous vascular muscle,\(^{23}\) the SHR, reasoning from larger SHR-sustained Ca\(^{2+}\) currents, would be expected to have lower Ca\(^{2+}\) activity (not higher) in this region.\(^{17}\) Thus, deficiencies of an intracellular mechanism (Ca\(^{2+}\) inactivation of sustained Ca\(^{2+}\) channels) by which vascular muscle cells are likely to regulate calcium for contraction are apparent. However, the functional significance of increased Ca\(^{2+}\) in newborn venous muscle cells for contraction or other than as a genetic membrane marker remains to be determined.

Increased SR Ca\(^{2+}\) release or reduced uptake might explain a large part of an amplified contraction with Ca\(^{2+}\) agonist. The action of the calcium antagonist is hypothesized to be a decreased influx and accentuated Ca\(^{2+}\) uptake by SR and removal at the surface membrane.\(^{2,24}\) Evidence for stimulation of uptake by SR is suggested by areas of very low calcium concentration immediately adjacent to the calcium release sites in SR and on the fragmentation
of regions of high calcium in the periphery of the cells at later times after stimulation (Figure 1). The Ca\(^{2+}\) increase at the periphery of the cell need not result in increased contraction amplitude because this region has relatively few contractile filaments.

From these results, we conclude that SHR show a greater maximum Ca\(^{2+}\) activity in response to the SdzS, with the the likelihood of an enhanced and prolonged SR calcium release that fits the prediction of a deficiency in calcium uptake. However, SHR are brought back to normal intracellular vascular muscle Ca\(^{2+}\) levels by SdzR (antagonist). If this is an opposite action on the same mechanism acted on by the agonist, all data would strengthen the suggestion of a deficiency of calcium regulation mechanisms in SHR. Considered along with evidence for decreased active calcium transport (uptake) in microsomes of cell membranes\(^{7,25,26}\) our observations of increased subcellular Ca\(^{2+}\) activity in vascular muscle cells of SHR make a compelling case for further exploration of cellular Ca\(^{2+}\) mechanisms in hypertension.

**References**


**KEY WORDS** • calcium • vascular smooth muscle cells • dihydropyridine • spontaneously hypertensive rats • Wistar-Kyoto rats
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