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# Calcium Waves in Unstimulated Left Ventricular Cardiomyocytes Isolated from Aged Spontaneously Hypertensive and Normotensive Rats

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**In this work, we described the incidence and the characteristics of calcium waves in cardiomyocytes isolated from aged normotensive rats (Wistar Kyoto, WKY) and age-matched spontaneously hypertensive rats (SHR) using imaging analysis of fura-2-loaded left ventricular cardiomyocytes. Left ventricular cardiomyocytes were isolated by enzymatic digestion from hearts of 18-20 month old WKY and aged-matched SHR. Intracellular calcium concentration did not differ in either strain, whereas the incidence of cells presenting calcium waves was greater in cardiomyocytes isolated from SHR. Moreover, cardiomyocytes isolated from SHR were significantly longer than those isolated from WKY. The calcium wave frequency was lower in SHR cardiomyocytes, while the velocity of the calcium waves was similar in both strains. Our results suggest that alterations in the calcium handling of SHR may contribute to the increased incidence of arrhythmias described in SHR hearts.** © 1997 Academic Press

In heart fragments and cardiac cells isolated from spontaneously hypertensive rats (SHR), an experimental model of cardiac hypertrophy, several electrophysiological and biochemical alterations have been described which are strictly dependent on alterations in intracellular calcium homeostasis. Although resting cytoplasmic calcium concentrations are similar in SHR isolated left ventricular cardiomyocytes and in the normotensive control Wistar Kyoto rat (WKY), SHR myocytes showed a larger calcium transient, associated with action potential, and increased SR calcium content [1]. However, in SHR decreased uptake of calcium by isolated SR fractions was found [2], while in the papillary

muscle after stimulation with isoproterenol, the loading of SR in SHR fibres is comparable to that in WKY [3]. Moreover, in experiments performed using patch-clamp technique we described an increase incidence of delayed after depolarization and membrane potential oscillations when experiments were performed in the absence of calcium chelator in the patch pipette. These delayed after depolarizations were likely associated to calcium waves observed at the end of a period of electrical field stimulation (4). Since calcium waves (i.e. a spontaneous, localised propagating increase in intracellular calcium) have been described in ventricular cardiomyocytes under several experimental conditions and different mechanisms may be responsible for the generation of calcium waves in unstimulated cardiomyocytes than those responsible for delayed after depolarization, in this work we studied the presence and characteristics of calcium waves in unstimulated single left ventricular cardiomyocytes isolated from aged, hypertrophied SHR hearts and age-matched WKY.

## METHODS

*Cell isolation.* This investigation confirms to the rules for the care and use of laboratory animals of the European Community. Eighteen- to twenty-month old spontaneously hypertensive rats (SHR) and age-matched normotensive control rats, Wistar Kyoto (WKY) were used for this study. Animals were obtained from Charles River (Italy). Left ventricular cardiomyocytes were isolated by enzymatic digestion. Hearts were removed from anaesthetised rats and perfused with gassed (95% O<sub>2</sub>: 5% CO<sub>2</sub>) Krebs-Henseleit solution (K-H) of the following composition (mM): 110 NaCl, 25 NaHCO<sub>3</sub>, 4.8 KCl, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 1.2 MgCl<sub>2</sub>, 11 mM D(+)-glucose, 10 taurine containing 1 mM CaCl<sub>2</sub>. The heart perfusion was followed for 5 min with nominally Ca<sup>2+</sup>-free K-H and then for 20 min with K-H containing 50 μM CaCl<sub>2</sub>, 0.1% type I collagenase (Sigma Chemical Company, St. Louis, Missouri, U.S.A.), 5 U/ml dispase (Type II, Boehringer Mannheim, Germany) and 0.1% bovine serum albumin fraction V, essentially fatty-acid free (BSA, Boehringer Mannheim). All the perfusion steps were performed at 37°C, while cell dispersion, purification and fura-2 loading were performed at room temperature (20-25°C). Fragments from left ventricle and septum were dispersed in K-H containing 100 μM CaCl<sub>2</sub> and 1% BSA. Cardiomyocytes were purified using a discontinuous gradient of BSA (4%), resuspended in K-H,

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TABLE 1

Incidence of "Calcium Waves" in Cardiomyocytes Isolated from the Left Ventricle of WKY and SHR

	No "calcium waves"	"Calcium waves"
WKY	57/96 (59%)	39/96 (41%)
SHR	68/151 (45%)	83/151* (55%)

Note. The number of analysed cells were 96 and 151 in WKY and SHR respectively. "Calcium waves" were defined as local variations of basal fluorescence ( $\geq$  than 15%).

\*  $P < 0.05$   $\chi^2$  test.

500  $\mu\text{M}$   $\text{CaCl}_2$  and loaded with 5  $\mu\text{M}$  fura-2AM (Molecular Probes, Eugene, Oregon, U.S.A.) for 45 min at room temperature. Cells were then washed and resuspended in K-H containing 1 mM  $\text{CaCl}_2$ . All solutions were gassed with 95%  $\text{O}_2$ : 5%  $\text{CO}_2$ . This method of myocyte preparation allowed us to obtain cell suspensions containing more than 70% of rod-shaped cardiomyocytes.

*Imaging analysis of cell fluorescence and cell length.* Experiments were performed blind. Fura-2 loaded cardiomyocytes were sedimented into a perfusion chamber placed on the platform of an inverted epifluorescence microscope (Nikon Diaphot, Japan) equipped with a 75 W Xenon lamp. Fluorescence images were collected with an intensified charge coupled device (CCD) video ISIS-M extended camera (Photonic Science, U.K.) at 340 or 380 nm excitation, emission 510 nm using an Image Analysis System "Magiscan" (Applied Imaging, New Castle, U.K.) and recorded on a videotape recorder (Sony U-Matic VO 9600-P, Japan) allowing a time resolution of 40 ms (video rate) and a long recording time. All cells found in an optical field (using  $20\times$  magnification objective) were analysed for at least 60 s. Experiments were performed at  $32^\circ\text{C}$ .

Fluorescence images were analysed using Imaging Analysis software "Tardis®" and custom-made software. All data were exported as ASCII file format and elaborated (MicroCal Origin® - 2.8 version). Calcium waves were identified as modifications of more than 15% of the basal fluorescence (gray mean) of the cell recorded at the single wavelength of 380 nm [5]. Cell length was measured; pixel/ $\mu\text{meter}$  calibration was achieved by a Burkert's chamber. For quantification of intracellular calcium, images were obtained at 340 and 380 nm and ratio was calculated on a pixel-by-pixel basis. Calibration curves were performed using ionomycin (Calbiochem, Inalco, Milan, Italy) and 2,4-butanedione monoxime (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) to prevent cell contraction in  $\text{Ca}^{2+}$ -overloaded myocytes [6]. All reagents were of analytical grade.

*Mathematic and statistical methods.* The principal frequency of calcium waves was obtained by Fast Fourier transform analysis. Values are presented as means  $\pm$  S.E.M.; when data were distributed asymmetrically, the median was also calculated. Statistical comparisons between data groups were performed by Student's t test or the Mann-Whitney test for two populations, as indicated. The  $\chi^2$  test ( $2\times 2$  contingency table) was used to compare the incidence of calcium waves in the two groups of cells. A P value  $\leq$  of 0.05 was considered significant.

## RESULTS AND DISCUSSION

In whole cells, the intracellular calcium concentrations were  $124 \pm 34.8$  nM ( $n=10$ ) and  $124 \pm 31.8$  nM ( $n=8$ ), respectively, in WKY and SHR left ventricular cardiomyocytes. Nor was the fluorescence intensity of whole cells (gray mean, measured with a similar CCD camera intensification) different in either strain ( $111.3$

$\pm 5.79$  A.U.,  $n=96$  and  $110.4 \pm 3.88$  A.U.,  $n=151$  in WKY and SHR respectively). Therefore, fura-2 loading of WKY and SHR isolated cardiomyocytes seems to be homogeneous, ruling out the possibility that a different signal/noise ratio can influence generation and/or detection of calcium waves. However, as shown in table 1, the majority of the SHR isolated cells presented spontaneous calcium waves, while calcium waves were detected in only 41% of WKY cardiomyocytes.

Cell lengths are reported in table 2: the SHR cells were significantly longer than those isolated from WKY (all analysed cells). Cell length was also measured in cardiomyocytes either presenting or not presenting calcium waves. As summarised in table 2, no statistically significant differences were found between the WKY and SHR cells in either group. Interestingly, in both strains, the mean cell length of the group presenting calcium waves was slightly longer than that of the group in which calcium waves were undetectable. In the case of WKY, this difference was at the limit of statistical significance ( $P=0.0535$ ). These data suggested that cells presenting calcium waves were not precontracted as expected for calcium-intolerant cardiomyocytes and therefore the generation of calcium waves does not depend on this experimental pathology.

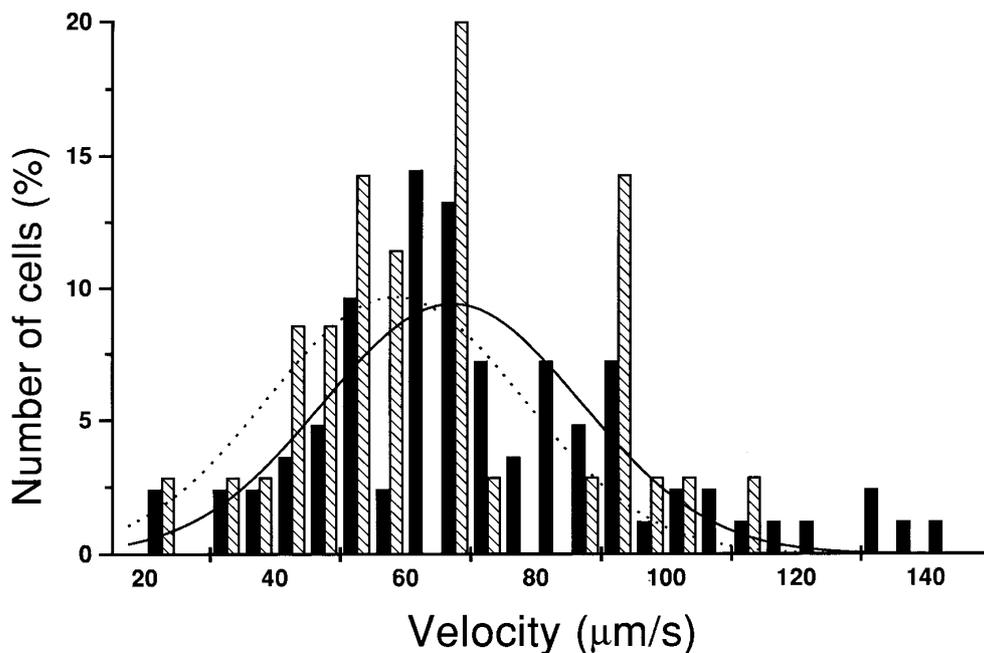
These results are in agreement with those reported by Brooksby et al. [1] in cardiomyocytes isolated from 16-week old WKY and SHR rats. These Authors report an identical calcium concentration in whole cardiomyocytes isolated from WKY and SHR rats as well as an increase in SHR cardiomyocyte length in comparison to WKY. The length of cardiomyocytes we isolated from 18-month old rats is greater in both WKY and SHR than that reported by Brooksby et al. [1], as expected, since our rats were older.

The distribution histogram of calcium wave velocity is shown in figure 1. In both WKY and SHR, velocity was a bell-shaped normal distribution ranging from 133 to 13  $\mu\text{m/s}$  in WKY and from 136 to 22  $\mu\text{m/s}$  in SHR. The mean velocity was very similar in WKY and SHR ( $64 \pm 3.7$   $\mu\text{m/s}$ ,  $n=39$  and  $72 \pm 2.8$   $\mu\text{m/s}$ ,  $n=83$  in WKY and SHR respectively). Thus the mechanism of calcium wave propagation in WKY and SHR cardiomyocytes might be quite similar.

TABLE 2  
Cell Length in WKY and SHR Cardiomyocytes

	Cell length ( $\mu\text{m}$ )	
	WKY	SHR
Analysed cells	$127 \pm 2.6$ (96)	$136 \pm 2.7^*$ (151)
No. "calcium waves"	$122 \pm 3.6$ (57)	$131 \pm 3.6$ (68)
"Calcium waves"	$133 \pm 3.3$ (39)	$140 \pm 4.0$ (83)

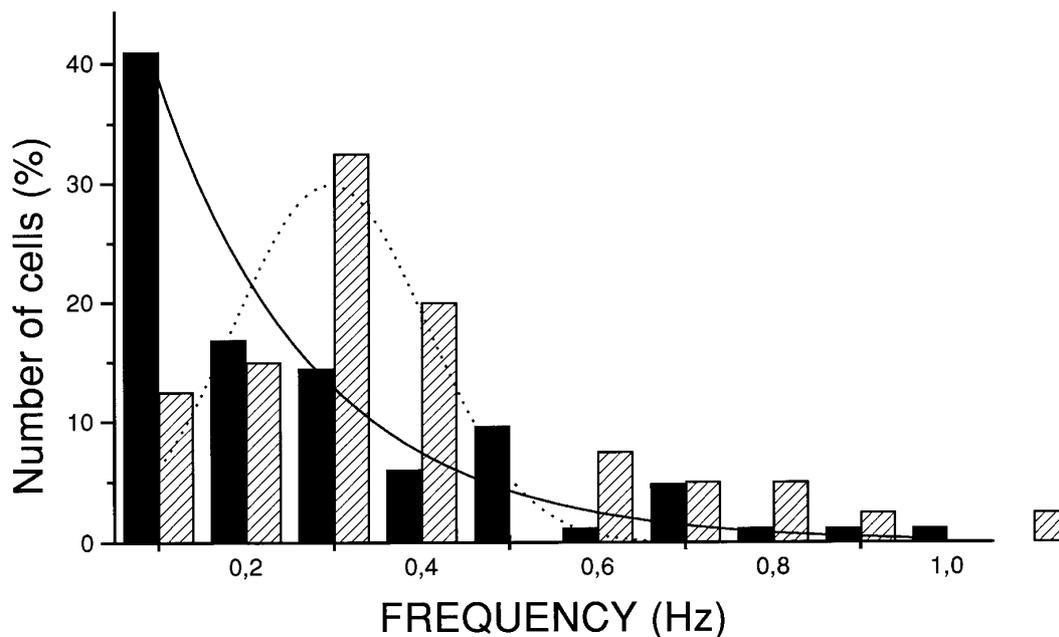
\*  $P < 0.02$  Student's t test. Values are the mean  $\pm$  S.E.M.; in parenthesis the number of cells.



**FIG. 1.** Distribution histogram of calcium wave velocity of WKY (hatched bars) and SHR (black bars) and Gaussian's fits (dot fit: WKY, line fit: SHR). Velocities were assembled with intervals of  $5 \mu\text{m/s}$  (horizontal scale). Vertical scale: percentage of cells as assembled according to horizontal axis interval.

The distribution histogram of calcium wave frequency is depicted in figure 2. The WKY frequency distribution is represented by a bell-shaped normal distribution, while the SHR frequency histogram is asym-

metric with a shift to the lower frequency of oscillation. The mean and the median were  $0.37 \pm 0.038$  and  $0.33$  ( $n = 39$ ) for WKY and  $0.25 \pm 0.024$  and  $0.18$  ( $n = 83$ ) for SHR,  $P < 0.012^*$  (Mann-Whitney test, two populations).



**FIG. 2.** Distribution histogram of calcium wave frequency of WKY (hatched bars) and SHR (black bars) and Gaussian's fits (dot fit: WKY, line fit: SHR). Frequencies were assembled with intervals of  $0.1 \text{ Hz}$  (Horizontal scale). Vertical scale: percentage of cells as assembled according to horizontal axis interval.

These data suggest that, as reported by Limas and Cohn [2], SR calcium uptake is decreased in the SHR heart and therefore more time is needed to replenish SR stores in SHR cardiomyocytes. Moreover, a localised increase in cytosolic calcium concentration which cannot be taken back up efficiently by the SR may induce calcium waves with a high incidence. Other explanations are also possible: the generation of calcium waves is dependent on several factors, including the sensitivity of an internal calcium detector and/or qualitative and quantitative changes in SR calcium release channels. In another model of hypertrophy, alterations in SR calcium release channels have already been described [7]. The frequency and velocity of calcium waves were related linearly in both strains ( $R = 0.46689$ ,  $P = 0.00469$  and  $R = 0.43515$ ,  $P = 0.00004$  in WKY and SHR respectively). Therefore in both WKY and SHR cardiomyocytes, the SR calcium loading influences not only the frequency of discharge (i.e. the probability of spontaneous release of a single SR element) but also the velocity of calcium wave propagation, as expected if each SR element is more or less near its discharge threshold.

In conclusion, our results show an increased incidence of calcium waves in cardiomyocytes isolated from the left ventricle of SHR as compared to those from WKY, while the intracellular calcium concentration and fluorescence intensity measured in whole cells are not statistically different. Because intracellular cal-

cium oscillations induce alterations in membrane potential [8], the high incidence of calcium waves in SHR cardiomyocytes may cooperate together with other calcium and electrophysiological alterations at the genesis of cardiac arrhythmias in SHR hearts [9].

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