Ca^{2+} Waves and Intracellular Ca^{2+} Concentration in Guinea Pig and Rat Myocytes

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SUMMARY

We measured [Ca^{2+}]i of guinea pig and rat myocytes with Ca^{2+} waves, using fura-2 fluorescence image processing. In guinea pig myocytes, Ca^{2+} waves were absent during the control perfusion period, but could be induced by the addition of strophanthidin (100 μM) or sodium cyanide (NaCN: 2 mM) to the perfusate. The [Ca^{2+}]i increased from the control values of 69 ± 5 nM and 46 ± 2 nM, to 263 ± 9 ($p < 0.05$ vs. control) nM and 225 ± 20 ($p < 0.05$) nM, respectively, when cells exhibited Ca^{2+} waves. Although 13% (16 of 121) of the rat myocytes displayed Ca^{2+} waves during the control perfusion, the [Ca^{2+}]i with Ca^{2+} waves (56 ± 9 nM) did not differ from [Ca^{2+}]i in the absence of Ca^{2+} waves (54 ± 3 nM). Ca^{2+} waves were induced by the perfusion with a high Ca^{2+} solution (24.5 μM) or NaCN (2 μM), and [Ca^{2+}]i increased from the control values of 67 ± 11 nM and 74 ± 5 nM, to 231 ± 41 ($p < 0.05$ vs. control) nM and 266 ± 64 nM, respectively, when cells exhibited Ca^{2+} waves. The Ca^{2+} waves were abolished by the removal of extracellular Ca^{2+}, or by the perfusion with ryanodine (10 μM) or caffeine (20 mM). In conclusion, it was shown that Ca^{2+} waves were due to oscillatory Ca^{2+} release and that the absolute value of [Ca^{2+}]i is important for the appearance of Ca^{2+} waves in guinea pig and rat myocytes. However, some rat myocytes with a control [Ca^{2+}]i level exhibited spontaneous Ca^{2+} waves during the control perfusion, showing a species difference in the susceptibility to oscillatory Ca^{2+} release from the sarcoplasmic reticulum. (Jpn Heart J **35**: 673–682, 1994)

Key words: Fura-2 Fluorescence digital processing Oscillatory Ca^{2+} release Sarcoplasmic reticulum Species difference

MANY cells display oscillations in intracellular Ca^{2+} concentration ([Ca^{2+}]i). Oscillations can arise from either the entry of external Ca^{2+} or from Ca^{2+} release from internal stores.[1] Spontaneous Ca^{2+} oscillation can occur in

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Received for publication January 24, 1994.

Accepted May 6, 1994.

unstimulated mammalian multicellular cardiac preparations and are manifest as periodic fluctuations in membrane voltage and current,[2] force,[3] or aequorin luminescence.[4,5] Under certain conditions, spontaneous Ca^{2+} release in mammalian cardiac myocytes takes the form of propagating changes in $[Ca^{2+}]i$, known as "Ca^{2+} waves".[6,7] Though the mechanisms of initiation and propagation of Ca^{2+} waves are uncertain, it is likely that Ca^{2+} waves are related to both oscillatory Ca^{2+} release and Ca^{2+}-induced Ca^{2+} release from the sarcoplasmic reticulum (SR).[7,8] Spontaneous Ca^{2+} oscillations have been implicated in the mechanism of certain arrhythmias and may produce a Ca^{2+} overload. For example, it has been shown that arrhythmias during digitalis intoxication[9] or ischemia/reperfusion[10,11] are due to a transient inward current caused by Ca^{2+} overload. Berlin et al[12] have shown that the transient inward current was often associated with Ca^{2+} waves.

Recently, it has been shown that contractile waves and sarcomere shortening are associated with Ca^{2+} transients, which can be directly detected by fluorescent Ca^{2+} probes such as fura-2[13] or indo-1.[14] In rat ventricular preparations, Ca^{2+} oscillations are observed when the extracellular Ca^{2+} concentration is in the physiological range, (1–2 mM), while ventricular preparations from most other species require some degree of Ca^{2+} overload.[15,16] However, there are few reports on the values of $[Ca^{2+}]i$ under control perfusion and during Ca^{2+} oscillations. Therefore, we examined the values of $[Ca^{2+}]i$ during Ca^{2+} overload to investigate how the changes in $[Ca^{2+}]i$ are associated with Ca^{2+} waves. We also measured the $[Ca^{2+}]i$ of guinea pig and rat myocyes to test for species differences in susceptibility to Ca^{2+} oscillations.

METHODS

Ventricular myocytes were isolated from male guinea pigs (250–300 g) or Wistar rats (200–250 g) following the method described by Hayashi et al.[17] The hearts were excised, attached to the bottom of a Langendorff column (60 cm height), and perfused with solutions gassed with 95% O_2–5% CO_2, and maintained at 37°C and pH 7.4. The first perfusate was Ca^{2+}-free Krebs solution to wash out the blood remaining in the heart cavities and coronary arteries. After 3–4 min of the initial perfusion, 50 m*l* of low $CaCl_2$ (50 μM) Krebs solution containing (1000 U/m*l* collagenase type V and 500 U/m*l* trypsin type III, (Sigma Chemical Co.) were added to the column and the perfusion was continued for 3–5 min. Finally, Ca^{2+}-free Krebs solution was introduced to wash out the residual enzyme solution from the heart.The ventricles were cut into small fragments with iris scissors, and myocytes were dispersed by gentle agitation in oxygenated Ca^{2+}-free Krebs solution. Finally, the Ca^{2+} concentration in the cell suspension was raised to 2.45 mM by the addition of $CaCl_2$. There was no difference in the

percent of rod-shaped, viable myoctes (40–50%) between guinea pigs and rats.

After isolation, cells were loaded with fura-2 by exposure to the acetoxymethyl ester, fura-2/AM, at a concentration of 5 μM, for 40 min. A small proportion of the fura-2 loaded cells was washed three times with modified Krebs solution. The cells were then placed in an experimental chamber, which was mounted on the stage of a Nikon TMD inverted microscope. After myocytes settled to the bottom of the chamber, the cells were perfused with modified Krebs solution (mM: NaCl 113.1, KCl 4.6, $CaCl_2$ 2.45, $MgCl_2$ 1.2, NaH_2PO_4 3.5, $NaHCO_3$ 21.9, and glucose 5), equilibrated with 95% O_2–5% CO_2 (pH 7.4) and maintained at 37°C. Myocytes selected for the study were rod-shaped with clear sarcomeres, sharp edges, and no blebs or granulations. The cells could be illuminated by transmitted or ultraviolet (UV) light, via an epifluorescence illuminator from a 300 W xenon lamp equipped with an interference filter. The fluorescence of fura-2 loaded cells was imaged using a Nikon Fluor (20×) objective. Video images were obtained using a silicon-intensified target camera (Hamamatsu Photonics K.K.), with the output digitized to a resolution of 512 × 512 pixels by an image processor (ARGUS; Hamamatsu Photonics K.K.). Images of fura-2 fluorescence ratios were then obtained by dividing, pixel by pixel, the 340 nm image after background subtraction, by the 380 nm image after background subtraction. The background did not contain cells. Since the autofluorescence of cells was about 1/10 of fura-2 fluorescence, it could be neglected. $[Ca^{2+}]i$ was measured in a 55 μm^2 field in each cell, and each fluorescence image was the accumulation of 60 successive video frames (2 sec/each collection).

We calculated $[Ca^{2+}]i$ by applying the ratio to the in vitro calibration curve.[18] The relationship between $[Ca^{2+}]i$ and the fura-2 fluorescence ratio was determined by adding fura-2 pentapotassium salt (50 μM) to small volumes of buffer: (K-MOPS 10, K_2H_2-EGTA +Ca-EGTA 10, NaCl 20, KCl 130); at pH 7.05, and 37°C. The dissociation constant (Kd) of EGTA is 214 nM.[19] The $[Ca^{2+}]i$ is related to the ratio of measured fluorescence signals excited at two excitation wavelengths, according to the following equation.[19]

$$[Ca^{2+}]i = Kd \times ß \times (R\text{-}R\ min)/(R\ max\text{-}R).$$

A high $[Ca^{2+}]o$ solution (24.5 mM) or a caffeine solution (20 mM) was applied without the adjustment of osmolarity, since high concentrations of sucrose (20–50 mM) did not affect the measurement of $[Ca^{2+}]i$ and Ca^{2+} waves. A paired or unpaired Student's t test was used to assess statistical significance, and $p < 0.05$ was considered significant. Data for all experiments are reported as mean ± SE.

Results

Some of the rat myocytes exhibited spontaneous contractile activities, and wavelike changes in fluorescence accompanied the propagating local contractions. This is called the "Ca^{2+} wave". The Table shows the percentage of cells which showed Ca^{2+} waves and the calculated $[Ca^{2+}]i$ during control perfusion (1–3 hours) at 37°C. Although guinea pig myocytes did not exhibit spontaneous Ca^{2+}

Table. The Percent of Cells with Ca^{2+} Waves and $[Ca^{2+}]i$ during Control Perfusion in Guinea Pig and Rat Myocytes

	Ca^{2+} waves (+)	$[Ca^{2+}]i$ (nM)
Guinea pig	0% (0/73 cells)	59 ± 5
Rat	13%* (16/121 cells)	54 ± 3 (without waves)
		56 ± 9 (with waves)

Values are mean ± SE. *$p < 0.05$ vs. guinea pig.

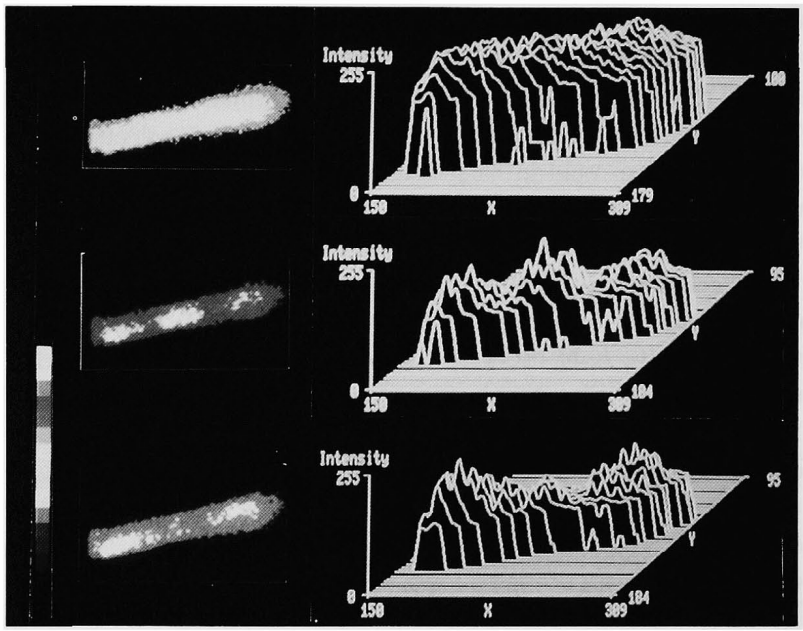

Figure 1. Multifocal spontaneous Ca^{2+} waves in a rat ventricular myocyte during the control perfusion period. The fluorescence emission intensity was measured at a 510 nm wavelength when the cell was excited at 380 nm. Pseudocolour images of $[Ca^{2+}]i$ (left) and the three-dimensional expression of the left images (right) are shown. The lower colour of the left bar and the lower pixels show higher $[Ca^{2+}]i$. The upper column is the interval between waves. Waves which originated from both ends of a cell move in opposite directions (the middle column) and collide (the lower column); further propagation of waves stopped.

waves, 16 out of 121 rat myocytes (13%) showed Ca^{2+} waves during the control perfusion period. The Table also shows that there was no difference in $[Ca^{2+}]i$ between the guinea pig and the rat myocytes (59 ± 5 nM; $n = 73$ vs. 54 ± 3 nM; $n = 105$). Further, the rat myocytes that showed spontaneous Ca^{2+} waves could not be identified on the basis of $[Ca^{2+}]i$.

There are two types of Ca^{2+} waves, a unifocal Ca^{2+} wave and multifocal Ca^{2+} waves. In a unifocal Ca^{2+} wave, a Ca^{2+} wave appeared at one end of a cell, and disappeared at another end of the cell.[18] Figure 1 shows typical multifocal Ca^{2+} waves in a rat ventricular myocyte during control perfusion. The wave of fluorescence consisted of an increased intensity at the 340 nm excitation wave length and decreased intensity at the 380 nm excitation wave length. The left column shows the emission intensities excited at 380 nm, and the right column is a three dimensional expression of the left column. We have chosen the fluorescence excited at 380 nm for clarity, since the changes at 380 nm were more marked than those at 340 nm. The upper column is the interval between waves. Waves which originated from both ends of a cell moved in opposite directions (the middle column), collided (the lower column), and further propagation of waves stopped. Spectral analysis showed that the propagation velocity of the waves was approximately 90 μm/sec.

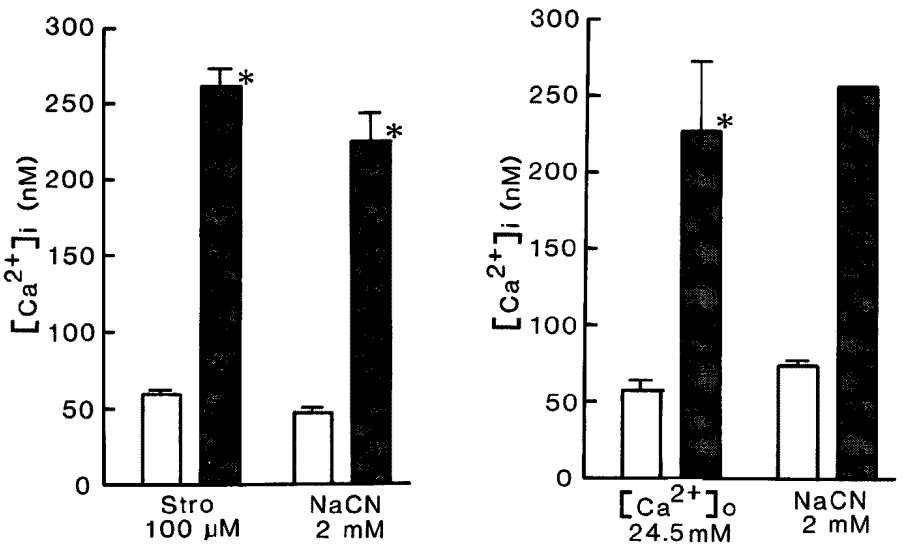

Figure 2. Left: The values of $[Ca^{2+}]i$ in guinea pig ventricular myocytes during the control perfusion (□) and during Ca^{2+} waves (■) elicited by either strophanthidin (100 μM) or sodium cyanide (NaCN: 2 mM). Values are means ± SE. *$p < 0.05$ vs. respective control. Right: The values of $[Ca^{2+}]i$ in rat ventricular myocytes during the control perfusion (□) and during Ca^{2+} waves (■) elicited by high Ca^{2+} solution (24.5 mM) or sodium cyanide (NaCN: 2 mM). Values are means ± SE. *$p < 0.05$ vs. control.

We examined the effects of drugs on Ca^{2+} waves to investigate the relationship between Ca^{2+} waves and $[Ca^{2+}]i$ under conditions of Ca^{2+} overload. Figure 2 (left) shows $[Ca^{2+}]i$ of guinea pig myocytes before and after the appearance of Ca^{2+} waves during perfusion with strophanthidin (Str) or sodium cyanide (NaCN). During the perfusion with 100 μM strophanthidin, 4 out of 15 cells exhibited Ca^{2+} waves at 5–15 min.[18] $[Ca^{2+}]i$ was measured when Ca^{2+} waves appeared, and the mean value of $[Ca^{2+}]i$ was 263 ± 9 nM, which was significantly higher than that of the control (69 ± 5 nM, $p < 0.05$). Though there were no changes in cell shape when Ca^{2+} waves appeared, most cells became shortened or rounded after variable time courses. There were no Ca^{2+} waves during the perfusion of strophanthidin in myocytes that were pretreated with 10 μM ryanodine (data not shown). After a 30 min perfusion with glucose-free Krebs solution containing 2 mM NaCN, the solution was washed out with Krebs solution containing 5 mM glucose. Six out of 28 cells exhibited Ca^{2+} waves during and after the NaCN perfusion.[20] The value of $[Ca^{2+}]i$ during Ca^{2+} waves was 225 ± 20 nM, which was significantly higher than that of the control (46 ± 2 nM, $p < 0.05$).

Figure 2 (right) shows the $[Ca^{2+}]i$ in rat myocytes before and after the appearance of Ca^{2+} waves during the perfusion with either high Ca^{2+} solution or NaCN. We used high $[Ca^{2+}]o$ solution instead of strophanthidin because rat hearts are insensitive to digitalis. During perfusion with high $[Ca^{2+}]o$ solution

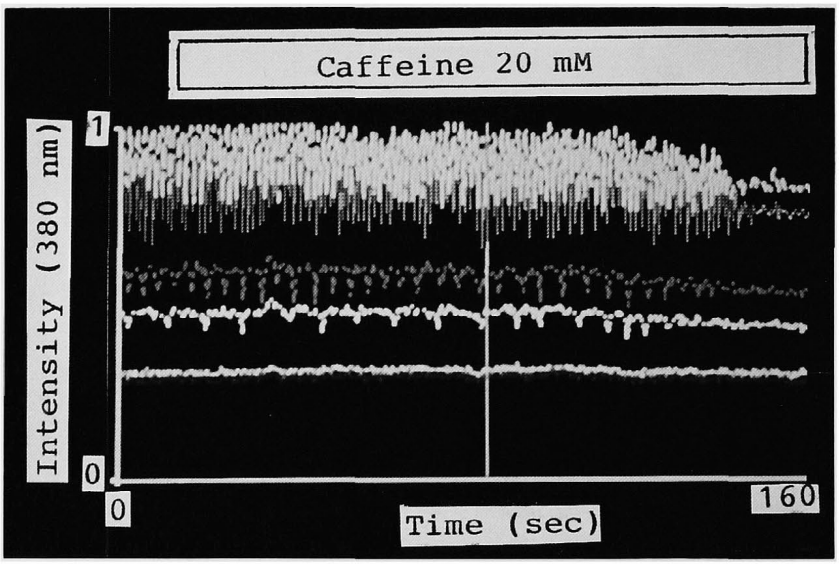

Figure 3. Effects of caffeine on Ca^{2+} waves of rat ventricular myocytes during control perfusion. Six cells were perfused with 20 mM caffeine. Ca^{2+} waves in 4 cells (from the upper cell to the fourth cell) disappeared within 2 min after the addition of caffeine. The fluorescence intensity was obtained at a 510 nm wavelength during excitation at 380 nm.

(24.5 mM), all 9 cells exhibited Ca^{2+} waves within 0.5–2 min. The value of $[Ca^{2+}]i$ during Ca^{2+} waves was 231 ± 41 nM, which was significantly higher than the control preparations (67 ± 11 nM, $p < 0.05$). When Ca^{2+}-free solution containing 1 mM EGTA was applied to myocytes with Ca^{2+} waves, Ca^{2+} waves disappeared and $[Ca^{2+}]i$ decreased to 40 ± 2 nM. When myocytes were perfused with glucose-free Krebs solution which contained 2 mM NaCN for 30 min, Ca^{2+} waves appeared and $[Ca^{2+}]i$ increased to 266 ± 64 nM from the control value of 74 ± 5 nM.

It has been reported that a high concentration of caffeine inhibits Ca^{2+} oscillations from the SR. Figure 3 shows a typical example in which rat ventricular myocytes with Ca^{2+} waves were subjected to 20 mM caffeine during control perfusion. Four cells showed changes in fluorescence intensity at 380 nm and the calculated $[Ca^{2+}]i$ varied with the wave frequency (280 nM and 1 Hz; 142 nM and 1.2 Hz; 85 nM and 0.5 Hz; 79 nM and 0.2 Hz). Within 2 min after the addition of caffeine, Ca^{2+} waves in 4 cells were all abolished. When 10 μM ryanodine was applied, Ca^{2+} waves disappeared in 5–6 min (data not shown).

Discussion

Ca^{2+} wave and fura-2 fluorescence microscopy: Spontaneous Ca^{2+} oscillations can occur in unstimulated mammalian multicellular cardiac preparations, which are manifest as periodic fluctuations in aequorin luminescence,[4,5] membrane voltage and current,[2] or force.[3] Scattered light fluctuations caused by microscopic myofilament motion has been also shown to be the result of Ca^{2+} oscillations.[21] The use of the fluorescent indicator, fura-2, in combination with an image processing allowed us to visualize Ca^{2+} waves with both spatial and temporal resolution. It is, however, necessary to recognize the limitations of the use of fura-2 and isolated myocyte preparations. There are several potential problems in using fura-2/AM, such as incomplete deesterification of fura-2/AM,[22] compartmentation of fura-2,[23] and photobleaching.[24]

Though there have been many reports on Ca^{2+} oscillations and Ca^{2+} waves, there are few measurements of $[Ca^{2+}]i$ during Ca^{2+} waves. Orchard et al[4] have reported that the value of $[Ca^{2+}]i$ in ferret papillary muscles was 15–40 μM under Ca^{2+} overload using aequorin. Cobbold et al[25] have reported localized elevations in $[Ca^{2+}]i$ of rat ventricular myocytes, during spontaneous Ca^{2+} release from the SR, could rise up to 1 μM. However, aequorin luminescence can overestimate $[Ca^{2+}]i$ during Ca^{2+} waves because of assumptions regarding the number of the cells containing aequorin and the dissynchronization of Ca^{2+} oscillation.[26] In this study, the mean value of $[Ca^{2+}]i$ during Ca^{2+} waves in guinea pig and rat myocytes was 225–266 nM. This value is 3–4 times the resting level of the con-

trol, which is compatible with those obtained using fura-2.[12,13,18] However, the spatial inhomogeneity in $[Ca^{2+}]i$ could interfere with the measurement of average $[Ca^{2+}]i$ in the presence of Ca^{2+} waves. In this study, each fluorescence image was the accumulation of 60 successive video frames (2 sec/each collection). Since the frequency of Ca^{2+} waves was 0.5–4 Hz in most cells, the motion artifact by spontaneous contractile activities of cells was thought to be largely eliminated. We, therefore, concluded that the increase in $[Ca^{2+}]i$ caused by Ca^{2+} oscillation could be expressed as the average increase in $[Ca^{2+}]i$ measured by fura-2 fluorescence.

A higher degree of spatial and temporal resolution is required though for the direct measurement of Ca^{2+} waves. Takamatsu and Wier[14] measured Ca^{2+} waves in voltage-clamped guinea pig myocytes using high-speed ratiometric images of Ca^{2+} waves. They used indo-1 as the Ca^{2+} indicator, since it undergoes a shift in peak emission wavelength upon binding Ca^{2+}.[19] The origin of Ca^{2+} waves in depolarized cells was detected as the spontaneous appearance of domelike regions of elevated $[Ca^{2+}]i$, approximtely 20 μm in diameter and 300 nM at the center. The propagation velocity of Ca^{2+} waves (90 μm/sec) in this study is compatible to the value of previous reports.[7,14] The observed spatial and temporal patterns of the origin and propagation of Ca^{2+} waves are consistent with the hypothesis that Ca^{2+} waves arise from a propagating Ca^{2+}-induced release of Ca^{2+}.[6,7]

Species difference: The first question is whether Ca^{2+} waves in rat myocytes during the control perfusion are physiological. Since guinea pig and rat myocytes were isolated under the same conditions and cell viability was similar in both animals (40–50%), it is likely that Ca^{2+} waves in rat myocytes represent normal responses of the SR to intracellular Ca^{2+} loading.

Spontaneous SR Ca^{2+} release has a marked species dependence. A sequence of the susceptibility of ventricular cells to spontaneous Ca^{2+} release was found to be as follows: rat>hamster>dog>cat>guinea pig>rabbit.[15,16] Lakatta et al[16] have described the species variations in the extracellular Ca^{2+} concentrations required for sarcolemmal light fluctuation using intact cardiac preparations. Thus, myocytes isolated from rats and guinea pigs were used to compare species difference in the conditions required for spontaneous Ca^{2+} release and the resultant Ca^{2+} waves. Previous reports in chemically skinned cells have shown that rat myocytes manifested periodic spontaneous contractions when bathed in a 100 nM Ca^{2+} solution, which is below the resting $[Ca^{2+}]i$ level.[27] These reports could explain why some rat myocytes showed Ca^{2+} waves in the control condition in this study. Furthermore, we could show that the occurrence of Ca^{2+} waves is unrelated to $[Ca^{2+}]i$ in isolated rat myocytes. The finding that Ca^{2+} waves were absent in guinea pig myocytes except under the conditions of Ca^{2+} overload is

also likely to be related to species difference to spontaneous SR Ca^{2+} release.

Effects of drugs which increase $[Ca^{2+}]i$: The Ca^{2+} overload is supposed to be a common pathway of various conditions such as digitalis intoxication, hypoxia/reoxygenation and ischemia/reperfusion. Cardiac glycosides are agents that produce a positive inotropic effect in cardiac muscles, by increasing $[Ca^{2+}]i$ via an inhibition of the Na^+/K^+ pump. A continued Na^+/K^+ pump inhibition has been shown to induce delayed aftercontractions and a transient inward current, which are caused by intracellular Ca^{2+} oscillations.[2] In guinea pig myocytes with Ca^{2+} waves, $[Ca^{2+}]i$ was higher than in control quiescent cells. Further, a ten-fold increase in $[Ca^{2+}]o$ concentration gave 3–4 fold increase in $[Ca^{2+}]i$[28] and induced Ca^{2+} waves. Finally, Ca^{2+} waves were abolished by the application of ryanodine or caffeine, which deplete Ca^{2+} stores of the SR.[4,5,16] Thus, it is likely that Ca^{2+} waves are related to the Ca^{2+} release from the SR.

Much attention has been focused on the role of Ca^{2+} in mediating or propagating ischemic cell injury. However, there is little direct evidence whether the increase in $[Ca^{2+}]i$ contributes to cell injury. This study showed that there is a significant increase in $[Ca^{2+}]i$, and that there are Ca^{2+} waves during and after the perfusion of NaCN. Since it has been reported that ATP is required for the SR Ca^{2+} oscillations,[29] it is likely that Ca^{2+} waves preceded ATP depletion during metabolic inhibition by NaCN. When strophanthidin, a high $[Ca^{2+}]o$ solution, or NaCN was applied, there were increases in $[Ca^{2+}]i$ and the appearance of Ca^{2+} waves, which suggested a relationship between Ca^{2+} waves and Ca^{2+} overload.

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