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Effect of osmotic stress on spontaneous calcium sparks in rat ventricular myocytes

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Key words

osmotic stress; calcium sparks; rat ventricular myocytes

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Abstract

Aim: To study whether the volume of cardiomyocytes and their functions would change under severe pathological conditions or osmotic stress. To clarify the role of ryanodine receptors/calcium release channels (RyRs) in the functional change, the effect of osmotic stress on spontaneous Ca^{2+} sparks in rat ventricular myocytes was investigated. **Methods:** A laser scanning confocal microscope was used to detect spontaneous Ca^{2+} sparks of intact or saponin permeabilized myocytes loaded with Fluo-4. High and low tonicity was obtained by adding sucrose and reducing NaCl concentration in the external medium, respectively. **Results:** In intact myocytes the frequency of Ca^{2+} sparks was increased and decreased by hyperosmotic (1.5 T) and hyposmotic (0.6 T) exposure, respectively. In addition, hyperosmotic exposure increased the temporal parameters and decreased the spatial parameter of Ca^{2+} sparks, while opposite changes occurred with hyposmotic exposure. The spatio-temporal properties of Ca^{2+} sparks were slightly affected by altering $[K^+]_i$ (50–200 mmol/L) in saponin permeabilized myocytes in the presence of 8% dextran. It was observed that the spatio-temporal parameters of the Ca^{2+} sparks in permeabilized myocytes were dose-dependently altered by dextran. The propagating velocity of Ca^{2+} waves in intact and permeabilized myocyte was also affected by osmotic pressure or dextran. **Conclusion:** The effect of osmotic stress on the frequency of spontaneous Ca^{2+} sparks might be ascribed to the change of myoplasmic Ca^{2+} and Ca^{2+} content in the sarcoplasmic reticulum, while the effect on the spatio-temporal properties is caused by the alteration of Ca^{2+} diffusion mainly resulting from the morphological change of the myocytes.

Introduction

The osmolarity of body fluids is tightly controlled under normal physiological conditions. But, the volume of the cells, including cardiomyocytes, can change under osmotic stress or in severe pathological states. For instance, after ischemia and reperfusion insult, cardiomyocyte swelling occurs[1]. The increase of the cell volume is also present in renal failure and after excessive water ingestion[2]. In contrast, cell shrinkage is observed with Na^+/K^+ pump inhibition and during apoptosis[3,4]. Furthermore, it has been shown that the increased blood tonicity in streptozotocin-induced diabetic rats leads to shrinkage of ventricular myocytes[5]. Many previous studies have indicated that both electrical and contractile properties of ventricular myocytes are affected by osmotic stress[1,5–9]. For example, it has been consistently found that L-type Ca^{2+} currents, Ca^{2+} transients and contraction evoked by action potential are decreased by prolonged exposure to hyposmotic medium[1,7].

It is well established that Ca^{2+} sparks are elementary events of Ca^{2+} release mediated by ryanodine receptors/Ca^{2+} release channels (RyRs) on the sarcoplasmic reticulum (SR) of muscle cells[10]. It is generally accepted that the Ca^{2+} transients evoked by action potential or depolarization are the spatial and temporal summation of many Ca^{2+} sparks. In resting ventricular myocytes, spontaneous Ca^{2+} sparks can

be detected. However, the physiological significance of these spontaneous events in cardiomyocytes is mostly unclear.

To our knowledge, there has been no study of the effect of osmotic stress on the spontaneous Ca^{2+} sparks in cardiomyocytes. Investigating this problem will be helpful to understand whether and how osmotic stress modulates RyR gating in cardiomyocytes. This study may also be relevant to further recognize the pathology of some diseases. For instance, it has been observed that the Ca^{2+} spark frequency of cardiomyocytes isolated from 5-week streptozotocin-induced diabetic rats significantly increased with respect to aged-matched control rats[11]. In addition, Ca^{2+} spark and Ca^{2+} transients exhibited a significantly prolonged duration in diabetic rats. An additional purpose of this study is that we have found that the aggregation of isolated RyRs of rabbit skeletal muscle was modulated by the concentration of K^+ and Na^+[12]. We wonder if such a modulation can be confirmed in intact myocytes through investigating the effect of osmotic stress on spontaneous Ca^{2+} sparks.

Materials and methods

Cell isolation and permeabilization Rat ventricular myocytes were isolated as described previously[13]. An adult Sprague-Dawley rat was anaesthetized by sodium pentobarbital (100 mg/kg, ip). The heart was quickly excised and perfused with O_2 saturated Ca^{2+}-free Tyrode's solution at 37 °C for 5 min. The perfusion solution was then switched to Tyrode's solution containing 1mg/mL collagenase (type I), 0.1 mg/mL trypsin (type I) and 0.1 mmol/L $CaCl_2$, and the heart was perfused for another 20 min. Afterwards, the ventricle was cut off, minced and filtered through nylon mesh. Isolated cells were washed with Ca^{2+}-free Tyrode's solution. $CaCl_2$ was then gradually added, and finally 1 mmol/L Ca^{2+} concentration was achieved. Isolated myocytes were kept in the standard physiological solution containing: NaCl 120 mmol/L, KCl 5.4 mmol/L, $MgCl_2$ 1.2 mmol/L, $CaCl_2$ 1 mmol/L, HEPES 20 mmol/L, glucose 15 mmol/L, pH 7.4 (NaOH) at room temperature (22–24 °C) for 1 h before use.

Isolated myocytes were permeabilized with 0.01% (*w/v*) saponin according to a modified method described in a previous study[14]. After permeabilization, the myocytes were perfused with saponin-free internal solution composed of: potassium aspartate 100 mmol/L, KCl 20 mmol/L, ATP 3 mmol/L, $MgCl_2$ 3.81 mmol/L, EGTA 0.5 mmol/L, $CaCl_2$ 0.1 mmol/L, phosphocreatine 10 mmol/L, creatine phosphokinase 5 U/mL, HEPES 10 mmol/L, Fluo-4 potassium salt 0.04 mmol/L, and 8% (*w/v*) dextran (MW 40 000); pH 7.2 (KOH). According to the computer program WinMAXC 2.5 (Stanford University, Stanford, CA, USA), free $[Ca^{2+}]$ and $[Mg^{2+}]$ in this solution were 43 nmol/L and 1 mmol/L, respectively. The total Ca^{2+} and Mg^{2+} necessary for obtaining different free $[Ca^{2+}]$ were also calculated with this program.

Detection of global Ca^{2+} transients and Ca^{2+} sparks Intact myocytes were loaded with 20 μmol/L Fluo-4 AM for 30 min at room temperature. A glass coverslip attached by the cells was mounted as the bottom of a chamber, which was put on the stage of an inverted microscope (Nikon Diaphot 300, Japan). Before taking records, the cells were superfused with standard physiological solution for 30 min for de-esterification of Fluo-4 AM. For permeabilized cells, the step of loading dye was omitted, and the dye Fluo-4 potassium salt was directly added into the bathing solution. Both global Ca^{2+} transients and Ca^{2+} sparks were observed by a laser scanning confocal microscope (MRC 1024, Bio-Rad, CA, USA) equipped with a ×60 oil-immersion objective (NA= 1.4). Fluo-4 was excited at a wavelength of 488 nm, and the fluorescence measured at >522 nm.

To monitor the global Ca^{2+} transients, the data of fluorescence intensity were collected at 1 Hz. Although the fluorescence signals were not calibrated in terms of Ca^{2+} concentration, the baseline fluorescence was used as a rough estimate of $[Ca^{2+}]_i$ and the changes in fluorescence signal represented alterations in $[Ca^{2+}]_i$. Caffeine-induced Ca^{2+} transients (CaTs) were evoked by repetitive exposures to 10 mmol/L caffeine for approximately 20 s at an interval of 10 min. The amplitude of F/F_0 was used as an estimate of SR Ca^{2+} content.

In order to record Ca^{2+} sparks, the fluorescence was recorded in either two-dimensional (X-Y) or line scan (X-T) mode. In the latter case, each scan line consisted of 512 pixels, and the length of the line was 84 μm (512 pixels× 0.164 μm/pixel). The scan line was oriented along the long axis of the myocyte, and the cell nuclei were avoided. A full image was obtained by stacking 512 scan lines. It took approximately 1 s (2 ms/line). The image obtained by line scan mode was processed by IDL software (Research Systems) and a modified spark detection algorithm[15]. Ca^{2+} sparks were identified as local peak elevations of fluorescent intensity (F), which were >3SD of the surrounding background levels (F_0). The measured parameters included amplitude (F/F_0), spatial full width at half maximum intensity (FWHM), full duration at half maximum intensity (FDHM), time to peak intensity (RT), and time for decay to half-peak intensity (THR). The frequency of Ca^{2+} sparks was represented by the number of Ca^{2+} sparks in one image obtained by line scan mode, event·S^{-1}·84 μm^{-1}.

To observe the effect of osmotic stress in intact ven-

tricular myocytes, the collection of Ca^{2+} sparks was started 10 min after changing the tonicity. The tonicity of standard physiological solution is referred to as ST. The tonicity in other solutions used in this study was 1 T (isosmotic solution), 0.6 T (hyposmotic solution) and 1.5 T or 2 T (hyperosmotic solution). Their osmolalities were measured with an osmometer. The compositions of these solutions are represented in Table 1.

Statistical analysis Statistical significance was determined by Student's *t*-test. $P<0.05$ was considered to be statistically significant.

Results

Effect of osmotic stress on spontaneous Ca^{2+} sparks in intact myocytes Several effects of osmotic stress on spontaneous Ca^{2+} sparks were found in this study. First, the frequency of spontaneous Ca^{2+} sparks was increased and decreased by hyper- and hypo-osmotic exposure, respectively. For instance, the frequency was increased from 1.80±0.25 event·S^{-1}·84 μm^{-1} to 3.55±0.27 event·S^{-1}·84 μm^{-1}, when the tonicity was enhanced from ST to 1.5 T. In another series of experiments it was found that the frequency in 1 T and 0.6 T were 5.11±0.52 event·S^{-1}·84 μm^{-1} and 1.31±0.12 event·S^{-1}·84 μm^{-1}, respectively. The effects of osmotic challenge on the frequency were mostly reversible (Table 2).

Besides the frequency, the spatio-temporal properties of Ca^{2+} sparks were also affected by osmotic stress. Representative records are shown in Figure 1A. The summarized results are displayed in Figure 1B and Table 2. As shown in Figure 1Ba, 1.5 T caused a left shift of FWHM distribution and a right shift of the distribution of the temporal parameters (RT and THR), respectively. The amplitude of Ca^{2+} sparks (F/F_0) was not affected (data not shown). The action of hyperosmotic exposure on the temporal parameters was reversible, while the spatial parameter was irreversibly affected.

Although increasing the frequency, 1 T had no influence on the spatio-temporal parameters of Ca^{2+} sparks (Table 2).

Table 1. Compositions of extracellular solutions.

Extracellular solution (mmol/L)	Standard physiological solution (ST)	Isosmotic solution (1 T)	Hyposmotic solution (0.6 T)	Hyperosmotic solution (1.5 T)	(2 T)
NaCl	120	64	64	120	120
KCl	5.4	5.4	5.4	5.4	5.4
$MgCl_2$	1.2	1.2	1.2	12	1.2
$CaCl_2$	1.0	1.0	1.0	1.0	1.0
HEPES	20	20	20	20	20
Glucose	15	15	15	15	15
Sucrose	0	103	0	140	280
Osmolality(mosm)	286	286	180	425	568

Table 2. Spatio-temporal properties and frequency of Ca^{2+} sparks in intact myocytes.

Extracellular solution	FWHM (μm)	FDHM (ms)	RT (ms)	THR (ms)	Frequency (event·S^{-1}·84 μm^{-1})
ST	1.99±0.02	26.10±0.33	9.25±0.14	18.10±0.26	1.80±0.25 (n=61)
1.5 T	1.74±0.01^c	37.05±0.39^c	11.90± 0.15^c	26.16±0.31^c	3.55±0.27 (n=101)^c
Reveral (1.5 T to ST)	1.75±0.02	26.44±0.71^f	9.50±0.30^f	18.58±0.60^f	0.89±0.14 (n=48)^f
1 T	2.03±0.01	26.81±0.28	9.34±0.10	19.23±0.24	5.11±0.52 (n=42)
0.6 T	2.12±0.02^c	22.05±0.20^c	8.43±0.09^c	15.49±0.17^c	1.31±0.12 (n=85)^c
Reveral (0.6 T to 1T)	1.93±0.03^f	27.39±0.34^f	9.91±0.16^f	19.25±0.29^f	3.64±0.43 (n=34) ^{f}
ST (10mmol/L Ca^{2+})	2.09±0.01	27.75±0.23	9.89±0.15	19.52±0.20	5.66±0.51 (n=61)^i
ST (16% dextran)	1.92±0.02	27.80±0.63	9.87±0.28	19.10±0.47	2.24±0.39 (n=28)

Ca^{2+} spark characteristics from different series of experiments were compared. n: the number of the examined myocytes. The number of spark events was ≥ 432 under various conditions. $^cP<0.01$, 1.5 T *vs* ST or 0.6 T *vs* 1 T. $^fP<0.01$, ST (reversal) *vs* 1.5 T or 1 T (reversal) *vs* 0.6 T. $^iP<0.01$, ST (+10 mmol/L [Ca^{2+}]) *vs* ST.

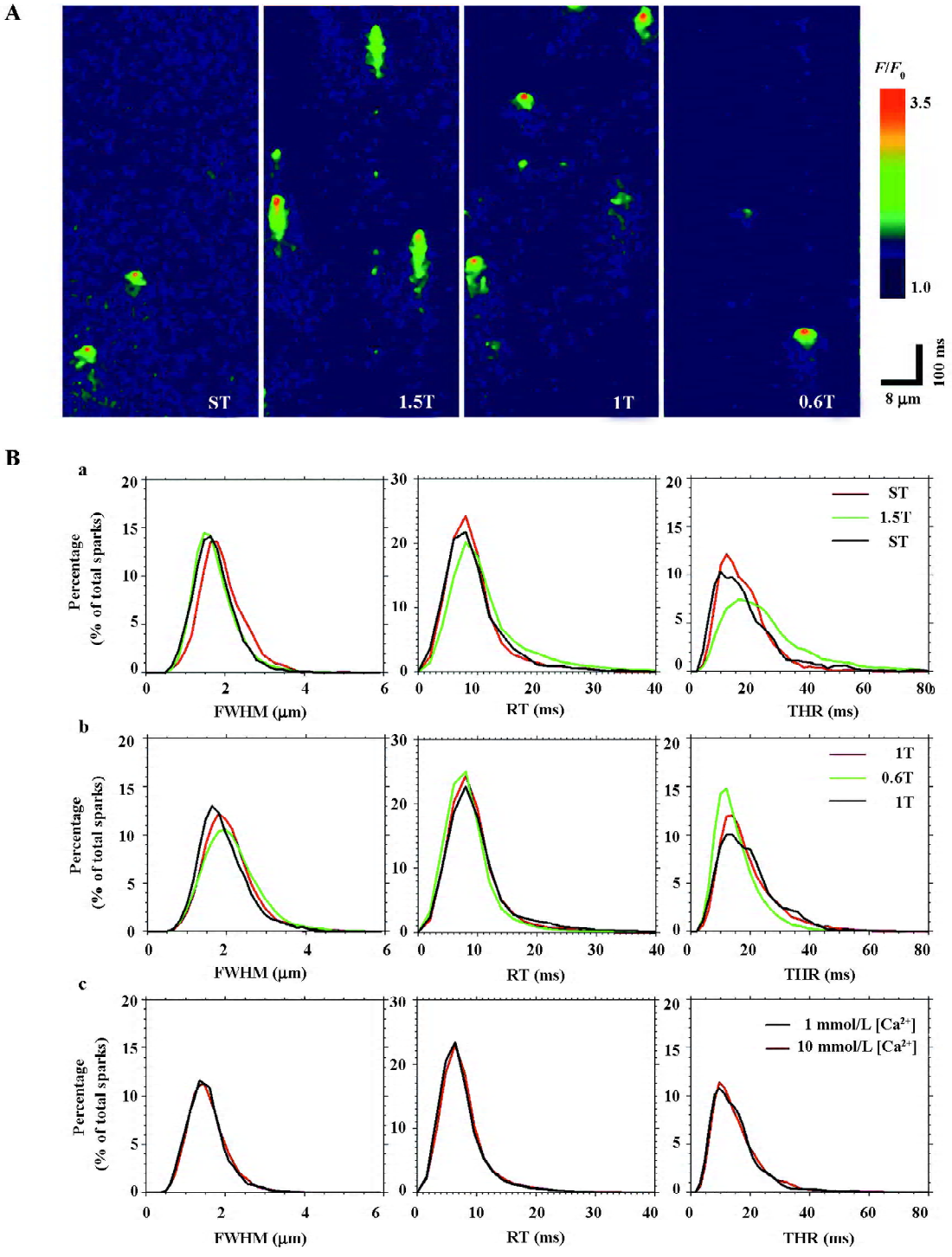

Figure 1. Effect of osmotic stress on spontaneous Ca^{2+} sparks in intact myocyte. A,representative line-scan images. B, profiles of characteristics of Ca^{2+} sparks, FWHM, RT and THR, under various tonicities (a,b) or at different $[Ca^{2+}]_o$ (c). Each curve was obtained from≥432 events (Ca^{2+} sparks).

Reducing the tonicity from 1 T to 0.6 T evidently caused the changes of the spatio-temporal parameters, which were just opposite to that induced by 1.5 T (Figure1Bb, Table 2). All of these effects of hyposmotic exposure were reversible.

Effect of osmotic stress on $[Ca^{2+}]_i$ and caffeine-induced Ca^{2+} transients in intact myocytes To explore if these effects of osmotic stress on spontaneous Ca^{2+} sparks result from the alterations of $[Ca^{2+}]_i$ and Ca^{2+} loading of the SR, the following experiments were performed.

First, in consistence with the previous studies[1,5–9], 1.5 T or 2 T caused an evident increase of resting $[Ca^{2+}]_i$ (Figure 2Aa), and this increase was eliminated in the absence of extracellular Ca^{2+} (Figure 2Ab). It is indicated that this increase of $[Ca^{2+}]_i$ induced by hyperosmotic challenge is due to Ca^{2+} influx. To see the effect of hyposmotic exposure, the myocytes were first perfused with isosmotic solution (1 T) for 10 min and then with hyposmotic solution (0.6 T).

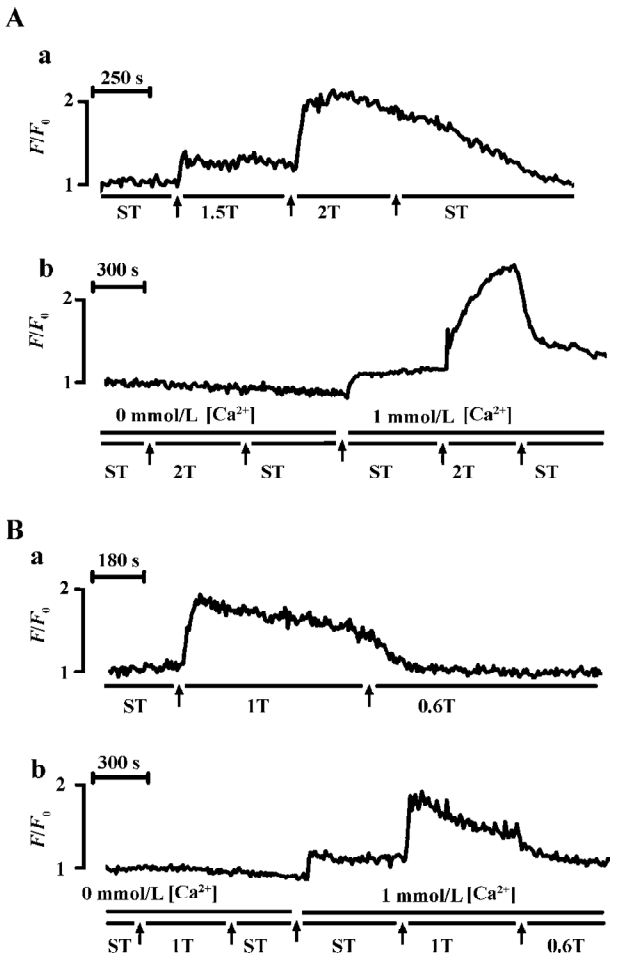

Figure 2. Effect of osmotic stress on $[Ca^{2+}]_i$ in intact myocyes. A, effect of hyper-osmotic (1.5T and 2T) stress (a) and its dependence on external Ca^{2+} ($[Ca^{2+}]_o$) (b). B, effect of isosmotic (1T) and hypo-osmotic (0.6T) stress (a) and its dependence on $[Ca^{2+}]_o$ (b)

Interestingly, it was observed that an increase of $[Ca^{2+}]_i$ occurred when extracellular solution was switched from ST to 1 T (Figure 2Ba). However, when the extracellular solution was further changed to 0.6 T, $[Ca^{2+}]_i$ was returned to the baseline (Figure 2Ba). Similarly, the increase of $[Ca^{2+}]_i$ induced by 1 T was absent in Ca^{2+} free extracellular solution (Figure 2Bb), indicating that it might be attributed to enhanced Ca^{2+} entry or reduced Ca^{2+} extrusion by Na^+-Ca^{2+} exchanger[1,7,16]. All of these effects of osmotic stress on $[Ca^{2+}]_i$ were reversible.

The effect of osmotic stress on CaTs was also investigated. The amplitude of the CaT was used as a rough estimate of Ca^{2+} loading of the SR. Figure 3A shows representative records of the CaT under various conditions. The summarized results displayed in Figure 3B illustrate that 1.5 T and 1 T significantly increased the CaT (124.5%±3.7% and 118.0%±3.4% of ST, respectively). In contrast, 0.6 T evidently decreased the CaT (83.7%±3.5% of 1 T or 98.8%± 4.2% of ST).

Afterwards, the action of increasing external Ca^{2+} ($[Ca^{2+}]_o$) was observed. In accordance with previous studies[17], elevating $[Ca^{2+}]_o$ to 10 mmol/L from 1 mmol/L resulted in an increase in the resting fluorescence (Figure 3Ad) and greatly augmented the CaT (135.4 %± 4.2% of ST). Correspondingly, the frequency of spontaneous Ca^{2+} sparks significantly increased at 10 mmol/L $[Ca^{2+}]_o$ (Table 2). However, raising $[Ca^{2+}]_o$ did not alter the spatio-temporal parameters (Figure 1Bc, Table 2).

Taken together, these results suggest that the change of the frequency induced by osmotic stress is attributed to the alteration of the $[Ca^{2+}]_i$ and Ca^{2+} loading of SR, while other factor(s) are responsible for the change of the spatio-temporal properties of Ca^{2+} sparks.

Effect of dextran, $[Ca^{2+}]_i$ and $[K^+]_i$ on spontaneous Ca^{2+} sparks in permeabilized myocytes Dextran is an uncharged, inert and highly branched polymer that can increase the viscidity of the solution and hence reduce the diffusibility of solute. To clarify the role of Ca^{2+} diffusion, the influence of dextran on the spatio-temporal properties of Ca^{2+} sparks was examined. As a control, adding 16% (*w/v*) dextran into external medium did not affect the spatio-temporal properties of Ca^{2+} sparks in intact myocytes (Table 2). However, in permeabilized myocytes the spatio-temporal properties of Ca^{2+} sparks were altered by dextran (Figure 4). In this study, 8% dextran was routinely used to prevent swelling of the permeabilized cells. It was found that, removing dextran from the internal solution significantly changed the spatio-temporal properties of Ca^{2+} sparks (Figure 4A,B). As shown in Figure 4B, the distribution of FWHM was evidently shifted

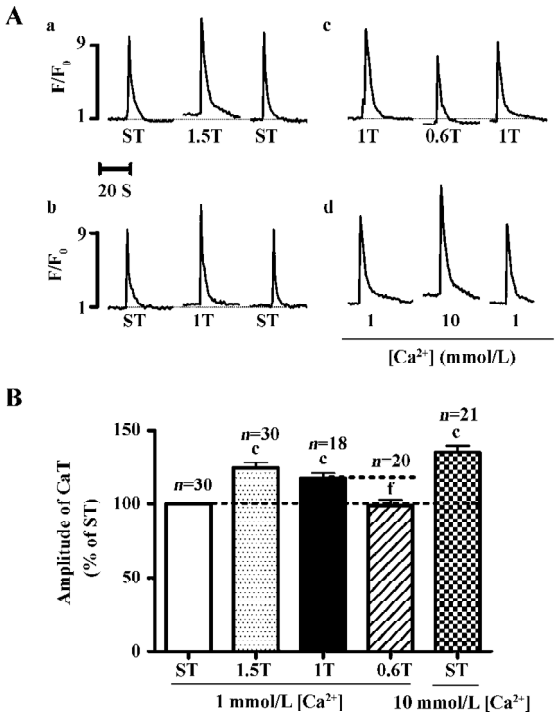

Figure 3. Effects of osmotic stress on caffeine induced Ca^{2+} transients (CaTs) in intact myocytes. Caffeine concentration: 10 mmol/L. A, representative records of the CaTs under various tonicities (a-c) and $[Ca^{2+}]_o$ (d). The CaTs in each panel were obtained from the same myocyte, and the interval between successive CaTs was 10 min. B, amplitude of the CaTs. n: the number of the examined myocytes. $^cP<0.01$ *vs* ST. $^fP<0.01$ *vs* 1 T.

to the right, while distributions of RT and THR were shifted to the left. Increasing dextran from 8% up to 16% had an opposite effect on these parameters (Figure 4B). The mean data are displayed in Table 3. The effect of dextran suggests the role of Ca^{2+} diffusion in determining the spatio-temporal properties of Ca^{2+} sparks.

To further confirm the role of $[Ca^{2+}]_i$ in modulating spontaneous Ca^{2+} sparks, the influence of $[Ca^{2+}]_i$ was investigated. It was revealed that raising $[Ca^{2+}]_i$ from 43 nmol/L to 75, 115 and 150 nmol/L increased the frequency from 3.19±0.14 to 6.13±0.34, 12.29±0.69 and 18.04±0.90 event·S^{-1}·84 μm^{-1}, respectively. Although the frequency was $[Ca^{2+}]_i$-dependent, the spatio-temporal parameters of Ca^{2+} sparks were not affected by moderate increase of $[Ca^{2+}]_i$ (data not shown).

In order to explore the role of ionic strength, spontaneous Ca^{2+} sparks were recorded from permeablized myocytes perfused with various concentrations of potassium aspartate ([K-Asp]) in the presence of 43 nmol/L $[Ca^{2+}]_i$. It was observed that the frequency at 50, 100, 150 and or 200 mmol/L [K-Asp] was 2.95±0.11, 3.20±0.15, 2.31±0.14 and 2.32±0.13 event·S^{-1}·84 μm^{-1}, respectively. It is indicated that high [K-Asp] might slightly reduce the frequency. However, increasing [K-Asp] from 50 mmol/L to 100 mmol/L had no detectable influence on the distribution of FWHM, RT and THR. However, further raising [K-Asp] up to 150 or 200 mmol/L slightly shifted these distribution curves to the left (Figure 4C).

Effect of osmotic stress and dextran on Ca^{2+} waves The action on Ca^{2+} waves of osmotic stress was observed in intact myocytes. It is known that the propagating velocity of the Ca^{2+} wave is proportional to the diffusion constant of Ca^{2+}[18]. Spontaneous Ca^{2+} waves only appeared in a small proportion of the myocytes. Figure 5A shows the representative images at different osmotic pressures. Summarized results are displayed in Figure 5B. It was observed that the velocity of Ca^{2+} waves was significantly decreased and increased by hyper- and hypo-osmotic exposure, respectively. Interestingly, the propagating velocity was not affected by 1 T (Figure 5B), although $[Ca^{2+}]_i$ and Ca^{2+} loading of the SR were increased (Figure 2B and 3Ab).

The effect of dextran on the propagating velocity of Ca^{2+} waves was investigated with permeabilized myocytes. Spontaneous Ca^{2+} waves could often be seen when $[Ca^{2+}]_i$ was elevated to 260 nmol/L (Figure 5C). The effect of dextran on the velocity of Ca^{2+} waves is represented in Figure 5D. Dextran-induced decrease of Ca^{2+} diffusion in permeabilized myocytes is clearly shown.

Discussion

It was observed in this study that both the frequency and spatio-temporal properties of spontaneous Ca^{2+} sparks in intact cells were affected by the tonicity of the extracellular solution. To understand the mechanisms underlying these effects, the role of the following factors is discussed individually: $[Ca^{2+}]_i$ and SR Ca^{2+} loading, ion strength, and Ca^{2+} diffusion.

Role of $[Ca^{2+}]_i$ and SR Ca^{2+} loading In this study clear evidence was obtained that the alteration of $[Ca^{2+}]_i$ and Ca^{2+} loading of the SR can account for the change of the frequency induced by osmotic stress but not for the change of the spatio-temporal properties of spontaneous Ca^{2+} sparks. First, exchanging 1T for ST or elevating $[Ca^{2+}]_o$ to 10 mmol/L increased $[Ca^{2+}]_i$ and SR Ca^{2+} loading (Figure 2 and 3). These actions only increased the frequency but did not change the spatio-temporal parameters of Ca^{2+} sparks in intact myocytes. Second, the frequency of Ca^{2+} sparks in permeablized myocytes was evidently increased by raising $[Ca^{2+}]_i$ from 43 nmol/L to 75 nmol/L, but the spatio-temporal parameters were not affected.

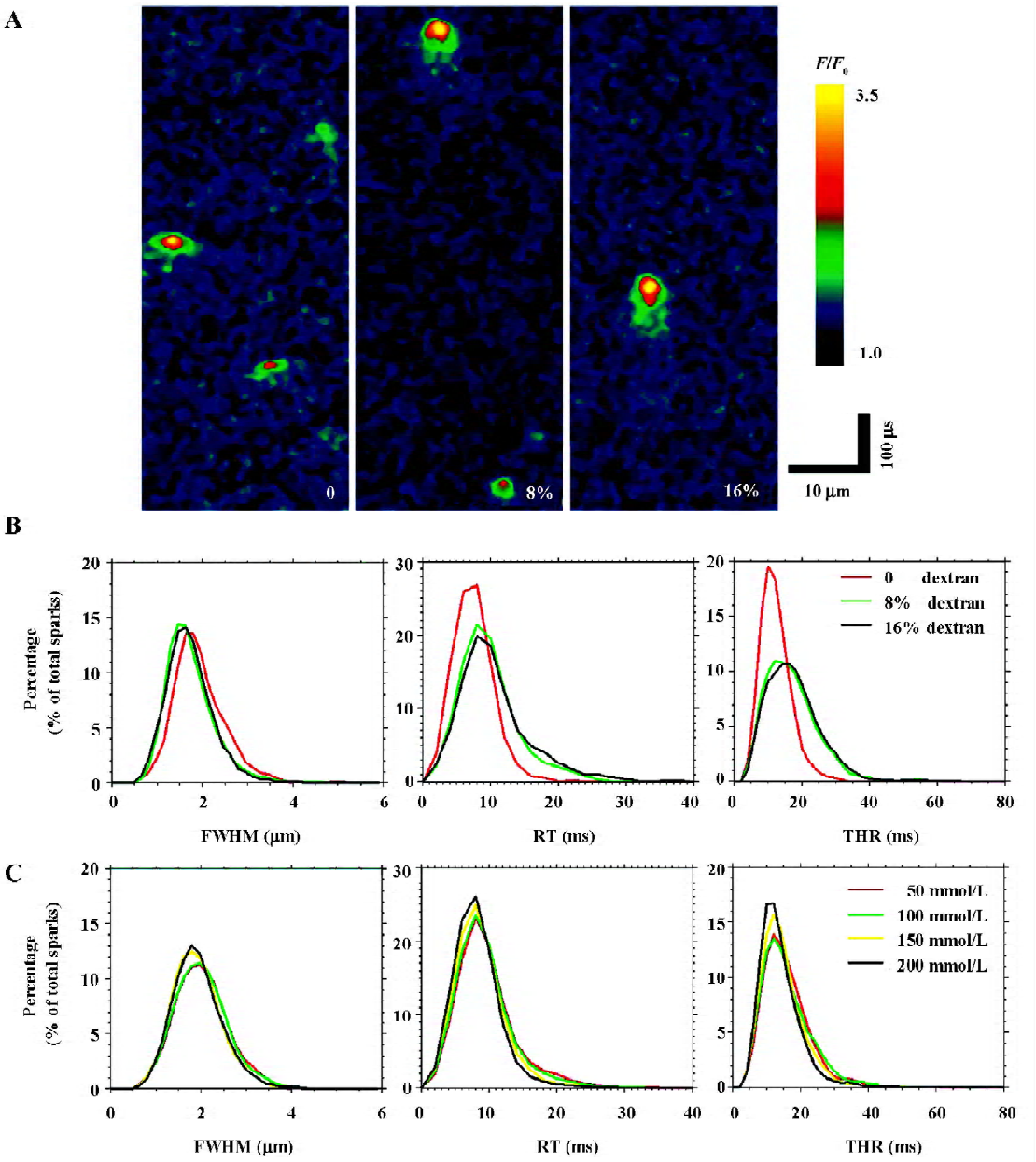

Figure 4. Effect of dextran and potassium aspartate on Ca^{2+} sparks in permeabilized myocytes. A, representative line-scan images at 0, 8% and 16% dextran. B and C, profiles of characteristics of Ca^{2+} sparks, FWHM, RT and THR, at various dextran concentrations (B) and [K-Asp] (C). Each curve was obtained from ≥634 events (Ca^{2+} sparks).

How are $[Ca^{2+}]_i$ and SR Ca^{2+} loading altered by osmotic stress? Several mechanisms may be involved. First, as the alteration of $[Ca^{2+}]_i$ induced by osmotic stress was dependent on extracellular Ca^{2+}, one mechanism is obviously Ca^{2+} entry. Previous studies have shown that the Ca^{2+} entry induced by osmotic stress are mediated by various channels, such as L-type Ca^{2+} channels[1,7,19] and stretch-activated channel[19,20]. Second, osmotic stress may change $[Ca^{2+}]_i$ and SR Ca^{2+} loading through altering cellular volume. Third, the alteration of $[Ca^{2+}]_i$ caused by the mechanisms just mentioned may be further affected through Ca^{2+}-induced Ca^{2+} release.

Role of ionic strength Previous studies have indicated that the activity of RyRs and the interaction between RyRs are modulated by ionic strength. For instance, with single-channel recording an increase of RyR activity has been shown with increasing KCl concentration[21,22]. By photon

correlation spectroscopy and atomic force microscopy we found that the aggregation of isolated RyRs of rabbit skeletal muscle is decreased by raising KCl concentration[12]. Accordingly, some properties of Ca^{2+} sparks would be expected to be influenced by the alteration of ionic strength.

It was found in this study that the frequency of spontaneous Ca^{2+} sparks in permeabilized myocytes was slightly decreased by raising [K-Asp] from 100 mmol/L to 150 or 200 mmol/L. This change seems to be different from the results obtained by single-channel recording[21,22]. The exact reason for this discrepancy is unclear. It may be because the Ca^{2+} spark is an event of Ca^{2+} release from a cluster of RyRs.

In addition, an evident difference is noted when comparing the effect of changing ion strength in internal solutions of permeabilized myocytes with the effect of changing the extracellular tonicity of intact myocytes. Raising [K-Asp] from 100 mmol/L to 200 mmol/L in permeabilized myocytes decreased THR by approximately 11%, whereas increasing the extracellular tonicity from ST to 1.5 T in intact myocytes remarkably increased THR by 45% (Table 2). Moreover, decreasing [K-Asp] from 100 mmol/L to 50 mmol/L had no effect on the spatio-temporal parameters, while decreasing the extracellular tonicity from 1 T to 0.6 T in intact myocytes increased FWHM by 4% and decreased THR by 20%. These results indicate that, if the alteration of ionic strength induced by osmotic stress is responsible for the change of the spatial parameter, it could not account for the change of the temporal parameters.

Role of Ca^{2+} diffusion Among the osmotic stress-induced changes of the spatio-temporal properties, the change of temporal parameters, especially THR is most conspicuous. The main factors contributing to the decline of the local $[Ca^{2+}]_i$ include closure of RyR, pumping Ca^{2+} back into the SR, Ca^{2+} diffusion and Ca^{2+} binding to buffers[23]. Even though Ca^{2+} uptake by the SR significantly influences the spatio-temporal parameters of Ca^{2+} sparks, Ca^{2+} diffusion is

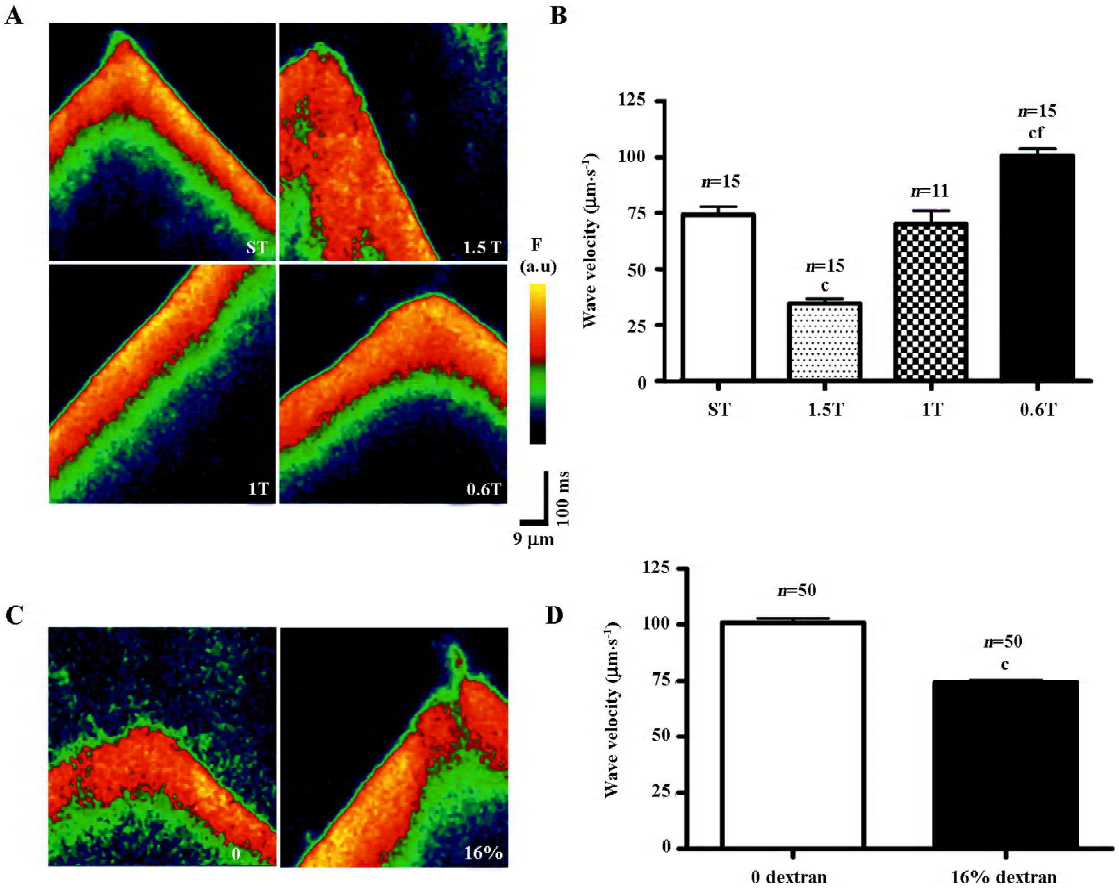

Figure 5. Effect of osmotic stress (A, B) and dextran (C, D) on propagating velocity of Ca^{2+} waves. A and C, representative line-scan images of Ca^{2+} waves in intact and permeabilized myocytes, respectively. B and D, propagating velocity of Ca^{2+} waves in intact and permeabilized myocytes, respectively. *n*: the recorded number of Ca^{2+} waves. $^{c}P<0.01$ *vs* ST or 0 dextran. $^{f}P<0.01$ *vs* 1 T.

proposed to be a dominant process in local $[Ca^{2+}]_i$ decline during Ca^{2+} sparks[23]. Hence, whether or not Ca^{2+} diffusion during Ca^{2+} sparks is altered by extracellular tonicity is questioned.

Previous observations have shown that transverse tubular-SR (T-SR) junction anatomy of amphibian skeletal muscle fibers, including the T-SR distance, was affected by extracellular tonicity[24,25]. The T-SR distance of about 16 nm was increased and decreased by hypo- and hyper-osmotic exposure, respectively. Unfortunately, information about the effect of the tonicity on the T-SR junction anatomy of ventricular myocytes is unavailable. If the effect in ventricular myocytes is similar, the T-SR distance of ventricular myocytes would be increased and decreased by hypo- and hyper-osmotic exposure, respectively. The T-SR distance is approximately 20 nm, while the spatial spread of Ca^{2+} sparks is over one micron. Therefore, the osmotic stress-induced alteration of the T-SR distance would affect Ca^{2+} diffusion during Ca^{2+} sparks and in turn the spatio-temporal parameters.

Besides the T-SR distance, other mechanisms may be involved in altering Ca^{2+} diffusion. For instance, accompanying cell shrinkage or swelling, the alteration in the Ca^{2+}-binding proteins' concentration would occur. It potentially would influence Ca^{2+} diffusion. In fact, our result showed that the propagating velocity of Ca^{2+} waves was increased and decreased by hypo- and hyper-osmotic exposure, respectively (Figure 5).

It was observed in this study that the propagating velocity of Ca^{2+} wave in permeabilized myocytes was significantly decreased by dextran (Figure 5). Furthermore, the FWHM of Ca^{2+} sparks in permeabilized myocytes was significantly reduced by dextran, whereas the temporal parameters, such as RT and THR, were remarkably increased (Figure 4B and Table 3). The effects of dextran on both Ca^{2+} waves and Ca^{2+} sparks in permeabilized myocytes are the same as the effects of hyperosmotic stress in intact myocytes. Therefore, it is likely that osmotic stress might affect Ca^{2+} diffusion and hence change the spatio-temporal properties of Ca^{2+} sparks in intact myocytes.

Role of other factors Besides the possible mechanisms mentioned above, other factors, such as SR Ca^{2+}-ATPase, Ca^{2+} release kinetics of RyR and control of RyR by dihydropyridine receptor (DHPR) control, may be involved.

In this study we used mag-fluo-4, a low affinity Ca^{2+} indicator (Kd =22 μmol/L), to directly measure the change of free Ca^{2+} concentration in the SR lumen. It was found that, following 10 mmol/L caffeine exposure, the rate of Ca^{2+} uptake back to the SR was not affected by osmotic stress (data not shown). In addition, it has been shown previously that, by completely blocking the SR Ca^{2+}-ATPase with thapsigargin, the time constant of $[Ca^{2+}]_i$ decline (τ) and FWHM of Ca^{2+} sparks increased 36% and 42%, respectively. On the contrary, stimulating the SR Ca^{2+}-ATPase by isoprenaline, the τ and FWHM decreased by 33% and 18%, respectively[23]. It was revealed in the present study that, increasing the tonicity from ST to 1.5 T remarkably enhanced THR by 45% and decreased FWHM by 13%, while decreasing the tonicity from 1 T to 0.6 T decreased THR by 20% and enhanced FWHM by 4%. Because the changes of the spatio-temporal properties of Ca^{2+} sparks induced by altering the tonicity or activity of the SR ATPase are qualitatively different, the role of the SR Ca^{2+}-ATPase may be excluded.

The RT of Ca^{2+} sparks may provide an estimate of Ca^{2+} release duration[26]. We have observed that the RT of Ca^{2+} sparks was increased by 29% and decreased by 10% at 1.5 T and 0.6 T, respectively. As the change in Ca^{2+} diffusion may also alter the RT, it is still uncertain whether or not osmotic stress affects the gating of RyR.

The previous study on the effect of osmotic stress on Ca^{2+} sparks in mice skeletal muscle fibers showed a robust and transient appearance of Ca^{2+} sparks when the fibers were returned to normal solution after being briefly exposed to hypotonic solution[27]. The initiation of Ca^{2+} sparks is proposed to result from osmotic shock-induced change of RyR gating controlled by DHPR. Differing from skeletal muscle

Table 3. Spatio-temporal properties and frequency of spontaneous Ca^{2+} sparks in permeabilized myocytes.

Internal solution	FWHM (μm)	FDHM (ms)	RT (ms)	THR (ms)	Frequency (event·S^{-1}·84 μm^{-1})
0 dextran	2.13±0.03	17.42±0.24	7.42±0.13	11.95±0.19	3.36±0.31 (n=20)
8% dextran	1.90±0.02^{c}	24.09±0.25^{c}	9.54±0.12^{c}	16.40±0.21^{c}	3.78±0.19 (n=46)^{c}
16% dextran	1.79±0.02^{cf}	25.55±0.40^{cf}	10.35±0.22^{c}	17.09±0.33^{cf}	3.17±0.17 (n=20)^{c}

n, number of the examined myocytes. The number of spark events was ≥634 at various dextran concentrations. $^{c}P<0.01$ *vs* 0 dextran. $^{f}P<0.01$ *vs* 8% dextran.

fibers, the effect of osmotic stress on Ca^{2+} sparks in ventricular myocytes maintained for an hour after osmotic challenge. Although the contact between RyR and DHPR in cardiac myocytes is not so close as in skeletal muscle fibers, the alteration in the T-SR distance may change DHPR-RyR coupling and hence Ca^{2+} sparks frequency.

Functional consequence of osmotic stress-induced change of Ca^{2+} sparks In cardiac myocytes, the electrical depolarization of the surface membrane leads to the influx of Ca^{2+} and then the Ca^{2+} release from the SR. As a result, the contraction of cardiomyocytes is evoked. Therefore, any fault in Ca^{2+} handling may influence the contractile function[28–31]. This study showed that in ventricular myocytes $[Ca^{2+}]_i$, Ca^{2+} loading in the SR and the frequency of spontaneous Ca^{2+} sparks enhanced with increasing the tonicity of extracellular solution. Moreover, the spatio-temporal properties of spontaneous Ca^{2+} spark and the propagating velocity of Ca^{2+} wave were also affected. These alterations induced by hyperosmotic stress may affect the contractile properties in various ways. For example, compared with that under normal or isosmotic condition, the time course of the contraction will be prolonged because of slower propagating of Ca^{2+} and slower decline of local $[Ca^{2+}]_i$. As a result of the increased $[Ca^{2+}]_i$, the relaxation may be incomplete. Opposite changes would be expected under hyposmotic stress. In fact, it has been reported that the time course (including the time to peak shortening and the time to half relaxation) of myocyte shortening in response to electrical stimulation decreases and increases by prolonged hypo- and hyper-osmotic exposures, respectively[1,5,7]. Besides, the alterations of $[Ca^{2+}]_i$ and the frequency of spontaneous Ca^{2+} sparks induced by osmotic stress may affect the pathways of the intracellular signal transduction. For instance, Ca^{2+} sparks in myocytes are able to yield mitochodrial Ca^{2+} uptake called Ca^{2+} marks, and in turn affect the cellular metabolism[32].

References

1 Brette F, Calaghan SC, Lappin S, White E, Colyer J, Le Guennec JY. Biphasic effects of hyposmotic challenge on excitation-contraction coupling in rat ventricular myocytes. Am J Physiol Heart Circ Physiol 2000; 279: H1963–71.

2 Vandenberg JI, Rees SA, Wright AR, Powell T. Cell swelling and ion transport pathways in cardiac myocytes. Cardiovasc Res 1996; 32 : 85–97.

3 Galvez AS, Ulloa JA, Chiong M, Criollo A, Eisner V, Barros LF, *et al.* Aldose reductase induced by hyperosmotic stress mediates cardiomyocyte apoptosis: differential effects of sorbitol and mannitol. J Biol Chem 2003; 278 : 38484–94.

4 Whalley DW, Hool LC, Ten Eick RE, Rasmussen HH. Effect of osmotic swelling and shrinkage on Na^+-K^+ pump activity in mammalian cardiac myocytes. Am J Physiol 1993; 265: C1201–10.

5 Howarth FC, Qureshi MA, White E. Effects of hyperosmotic shrinking on ventricular myocyte shortening and intracellular Ca^{2+} in streptozotocin-induced diabetic rats. Pflugers Arch 2002; 444: 446–51.

6 Lado MG, Sheu SS, Fozzard HA. Effects of tonicity on tension and intracellular sodium and calcium activities in sheep heart. Circ Res 1984; 54: 576–85.

7 Li GR, Zhang M, Satin LS, Baumgarten CM. Biphasic effects of cell volume on excitation-contraction coupling in rabbit ventricular myocytes. Am J Physiol Heart Circ Physiol 2002; 282: H1270–7.

8 Ogura T, You Y, McDonald TF. Membrane currents underlying the modified electrical activity of guinea-pig ventricular myocytes exposed to hyperosmotic solution. J Physiol 1997; 504: 135–51.

9 Mizutani S, Prasad SM, Sellitto AD, Schuessler RB, Damiano RJ, Jr, Lawton JS. Myocyte volume and function in response to osmotic stress: observations in the presence of an adenosine triphosphate-sensitive potassium channel opener. Circulation 2005; 112: I219–23.

10 Shirokova N, Gonzalez A, Kirsch WG, Rios E, Pizarro G, Stern MD, *et al.* Calcium sparks: release packets of uncertain origin and fundamental role. J Gen Physiol 1999; 113: 377–84.

11 Yaras N, Ugur M, Ozdemir S, Gurdal H, Purali N, Lacampagne A, *et al.* Effects of diabetes on ryanodine receptor Ca release channel (RyR2) and Ca^{2+} homeostasis in rat heart. Diabetes 2005; 54: 3082–8.

12 Hu XF, Chen KY, Xia R, Xu YH, Sun JL, Hu J, *et al.* Modulation of the interactions of isolated ryanodine receptors of rabbit skeletal muscle by Na^+ and K^+. Biochemistry 2003; 42: 5515–21.

13 Zhu HF, Dong JW, Zhu WZ, Ding HL, Zhou ZN. ATP-dependent potassium channels involved in the cardiac protection induced by intermittent hypoxia against ischemia/reperfusion injury. Life Sci 2003; 73: 1275–87.

14 Lukyanenko V, Gyorke S. Ca^{2+} sparks and Ca^{2+} waves in saponin-permeabilized rat ventricular myocytes. J Physiol 1999; 521: 575–85.

15 Cheng H, Song LS, Shirokova N, Gonzalez A, Lakatta EG, Rios E, *et al.* Amplitude distribution of calcium sparks in confocal images: theory and studies with an automatic detection method. Biophys J 1999; 76: 606–17.

16 Shacklock PS, Wier WG, Balke CW. Local Ca^{2+} transients (Ca^{2+} sparks) originate at transverse tubules in rat heart cells. J Physiol 1995; 487: 601–8.

17 Lukyanenko V, Gyorke I, Gyorke S. Regulation of calcium release by calcium inside the sarcoplasmic reticulum in ventricular myocytes. Pflugers Arch 1996;4 32: 1047–54.

18 Keizer J, Smith GD, Ponce-Dawson S, Pearson JE. Saltatory propagation of Ca^{2+} waves by Ca^{2+} sparks. Biophys J 1998; 75: 595–600.

19 Smogorzewski M, Galfayan V, Massry SG. High glucose concentration causes a rise in $[Ca^{2+}]_i$ of cardiac myocytes. Kidney Int 1998; 53: 1237–43.

20 Wright AR, Rees SA. Cardiac cell volume: crystal clear or murky waters? A comparison with other cell types. Pharmacol Ther 1998; 80: 89–121.

21 Meissner G, Rios E, Tripathy A, Pasek DA. Regulation of skel-

etal muscle Ca^{2+} release channel (ryanodine receptor) by Ca^{2+} and monovalent cations and anions. J Biol Chem 1997; 272: 1628–38.
22 Liu W, Pasek DA, Meissner G. Modulation of Ca(2+)-gated cardiac muscle Ca(2+)-release channel (ryanodine receptor) by mono- and divalent ions. Am J Physiol 1998; 274: C120–8.
23 Gomez AM, Cheng H, Lederer WJ, Bers DM. Ca^{2+} diffusion and sarcoplasmic reticulum transport both contribute to $[Ca^{2+}]_i$ decline during Ca^{2+} sparks in rat ventricular myocytes. J Physiol 1996; 496: 575–81.
24 Martin CA, Petousi N, Chawla S, Hockaday AR, Burgess AJ, Fraser JA, *et al*. The effect of extracellular tonicity on the anatomy of triad complexes in amphibian skeletal muscle. J Muscle Res Cell Motil 2003; 24: 407–15.
25 Chawla S, Skepper JN, Hockaday AR, Huang CL. Calcium waves induced by hypertonic solutions in intact frog skeletal muscle fibres. J Physiol 2001; 536: 351–9.
26 Wang SQ, Song LS, Xu L, Meissner G, Lakatta EG, Rios E, *et al*. Thermodynamically irreversible gating of ryanodine receptors in situ revealed by stereotyped duration of release in Ca(2+) sparks. Biophys J 2002; 83: 242–51.
27 Wang X, Weisleder N, Collet C, Zhou J, Chu Y, Hirata Y, *et al*. Uncontrolled calcium sparks act as a dystrophic signal for mammalian skeletal muscle. Nat Cell Biol 2005; 7: 525–30.
28 Wehrens XH, Lehnart SE, Marks AR. Intracellular calcium release and cardiac disease. Annu Rev Physiol 2005; 67: 69–98.
29 Yao A, Su Z, Nonaka A, Zubair I, Spitzer KW, Bridge JH, *et al*. Abnormal myocyte Ca^{2+} homeostasis in rabbits with pacing-induced heart failure. Am J Physiol 1998; 275: H1441–8.
30 Braunwald E, Bristow MR. Congestive heart failure: fifty years of progress. Circulation 2000; 102: IV14–23.
31 Wehrens XH, Marks AR. Novel therapeutic approaches for heart failure by normalizing calcium cycling. Nat Rev Drug Discov 2004; 3: 565–73.
32 Pacher P, Thomas AP, Hajnoczky G. Ca^{2+} marks: miniature calcium signals in single mitochondria driven by ryanodine receptors. Proc Natl Acad Sci USA 2002; 99: 2380–5.