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Intracellular free zinc during cardiac excitation–contraction cycle: calcium and redox dependencies

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Aims Zinc exists in biological systems as bound and histochemically reactive free Zn^{2+}. It is an essential structural constituent of many proteins, including enzymes from cellular signalling pathways, in which it functions as a signalling molecule. In cardiomyocytes at rest, Zn^{2+} concentration is in the nanomolar range. Very little is known about precise mechanisms controlling the intracellular distribution of Zn^{2+} and its variations during cardiac function.

Methods and results Live-cell detection of intracellular Zn^{2+} has become feasible through the recent development of Zn^{2+}-sensitive and –selective fluorophores able to distinguish Zn^{2+} from Ca^{2+}. Here, in freshly isolated rat cardiomyocytes, we investigated the rapid changes in Zn^{2+} homeostasis using the Zn^{2+}-specific fluorescent dye, FluoZin-3, in comparison to Ca^{2+}-dependent fluo-3 fluorescence. Zn^{2+} sparks and Zn^{2+} transients, in quiescent and electrically stimulated cardiomyocytes, respectively, were visualized in a similar manner to known rapid Ca^{2+} changes. Both Zn^{2+} sparks and Zn^{2+} transients required Ca^{2+} entry. Inhibiting the sarcoplasmic reticulum Ca^{2+} release or increasing the Ca^{2+} load in a low-Na^{+} solution suppressed or increased Zn^{2+} movements, respectively. Mitochondrial inhibitors slightly reduced both Zn^{2+} sparks and Zn^{2+} transients. Oxidation by H_2O_2 facilitated and acidic pH inhibited the Ca^{2+}-dependent Zn^{2+} release.

Conclusion It is proposed that Zn^{2+} release during the cardiac cycle results mostly from intracellular free Ca^{2+} increase, triggering production of reactive oxygen species that induce changes in metal-binding properties of metallothioneins and other redox-active proteins, aside from ionic exchange on these proteins.

Keywords Redox regulation • Intracellular free zinc • Excitation–contraction coupling • Intracellular calcium homeostasis • Cardiomyocyte

1. Introduction

Zinc is second to iron, the most abundant transition metals in the body. It is an important nutrient, being an essential catalytic or structural component in thousands of enzymes involved in cellular signalling pathways and transcription factors. It may also function as a signalling molecule, such as a neurotransmitter.[1] Total zinc in eukaryotic cells (up to 200 μM) is not very different from calcium, with 30% localized in the nucleus, 50% in the cytosol and organelles and the remainder associated with proteins.[2] In cardiomyocytes, the intracellular free Zn^{2+} concentration, $[Zn^{2+}]_i$, has been measured to be less than one nanomolar in physiological conditions,[3] similar to those reported in HT-29 cells,[4] i.e. some 100-fold less than $[Ca^{2+}]_i$. Moreover, oxidants caused a roughly 30-fold increase in $[Zn^{2+}]_i$ but only two-fold in $[Ca^{2+}]_i$ in cardiomyocytes.[3]

Zinc homeostasis in mammalian cells results from a co-ordinated regulation by different proteins involved in uptake, excretion and intracellular storage/trafficking of Zn^{2+}.[5] Zn^{2+} influx is facilitated by ZIP (solute-linked carrier, SLC39) proteins[6] and occurs through different channels, including the L-type Ca^{2+} channels in heart cells.[7] The efflux of Zn^{2+} or its sequestration is promoted by the ZnT (SLC30) families of transporters.[8] Furthermore, a Na^{+}–Zn^{2+} exchange mechanism different from ZnT1 and Na^{+}–Ca^{2+} exchange mediates Zn^{2+} extrusion in HEK293 cells and cortical neurons.[9] A Zn^{2+}–H^{+} antiport mechanism could be responsible for Zn^{2+} accumulation in intracellular organelles.[10] Intracellular Zn^{2+} is

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thought to be in equilibrium with many soluble cytosolic Zn^{2+}-binding proteins, such as metallothionein/thionein. The apoprotein can bind a total of seven Zn^{2+} ions in two domains co-ordinated by several cysteine ligands. In addition, free Zn^{2+} is modulated by the redox state and the level of reactive oxygen species (ROS) in cells,[11] as was reported early in cardiomyocytes.[3] Zinc/thiolate clusters in metallothionein bind Zn^{2+} rather tightly and yet can make it available, providing a mechanism whereby Zn^{2+} availability and redox metabolism are linked.[11] About 30% of the Zn^{2+}-buffering capacity is due to sulfur donors (thiols) and thus serves at the same time as redox (oxidant) buffer capacity.[11]

Zinc is known to induce Ca^{2+}–calmodulin-dependent protein kinase II autophosphorylation,[12] to inhibit protein tyrosine phosphatases[13] and voltage-gated Ca^{2+} channels in mammals,[14] and to be highly cytotoxic.[15] In cardiomyocytes, it is known that $[Zn^{2+}]_i$ can be greatly increased by thiol-reactive oxidants, and free Zn^{2+} may contribute to oxidant-induced alterations of excitation–contraction coupling.[3] Recently, aside from Zn^{2+} release after metallothionein nitrosylation,[16] Jang *et al.*[17] reported that NO mobilizes intracellular Zn^{2+} in cardiac tissue via the cGMP–protein kinase G signalling pathway, implicating the mitochondrial ATP-sensitive K^+ channel. In addition, Zn^{2+} release, a phenomenon named 'Zn^{2+} wave', originating from the endoplasmic reticulum and/or the surrounding area in mast cells after cross-linking of the high-affinity immunoglobulin E receptor, was shown to be dependent on Ca^{2+} influx without any contribution of extracellular Zn^{2+}.[18]

Based on the multiple functions of Zn^{2+}, it is tempting to hypothesize a crucial role of intracellular free Zn^{2+} in modulating excitation–contraction coupling of cardiomyocytes in physiological conditions, besides its detrimental roles in physiopathological conditions. However, very little is known about the precise mechanisms that control the cellular distribution of Zn^{2+} in cardiomyocytes, or its variations during the cardiac cycle. Live-cell detection of intracellular Zn^{2+} has become feasible only through the recent development of Zn^{2+}-sensitive and –selective fluorophores, which are able to distinguish Zn^{2+} from Ca^{2+}.[19,20] Therefore, we aimed to investigate the rapid changes in Zn^{2+} homeostasis during the excitation–contraction cycle of cardiomyocytes in physiological conditions by using a Zn^{2+}-specific fluorescent dye, FluoZin-3. We demonstrated that rapid changes in free Zn^{2+} mostly result from Zn^{2+} displacement by Ca^{2+} ions from intracellular binding sites that are highly sensitive to the redox status of the cardiomyocytes.

2. Methods

All animals were handled in accordance with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996). The protocol was approved by the Ankara University, with reference number 2007-15-44.

2.1 Cell isolation

Rats were anaesthetized using sodium pentobarbital (30 mg/kg, intraperitoneal). Ventricular cardiomyocytes were isolated from adult male 3-month-old Wistar rats (200–250 g) by using a standard enzymatic digestion, as mentioned previously.[21] Only rod-shaped cells, quiescent, and excitable by field stimulation, were used for experiments.

2.2 Microspectrofluorimetric studies

Fluorescence changes were recorded using a microspectrophotometer and FELIX software (PTI, Lawrenceville, NJ, USA) in cardiomyocytes loaded with cell-permeable acetoxymethoxy derivatives of FluoZin-3 or fluo-3 (FluoZin-3-AM or fluo-3 AM; 35–40 min incubation) and excited at 0.2 Hz by field stimulation using two platinum electrodes positioned on either side of the recording chamber. The basal levels of $[Zn^{2+}]_i$ and $[Ca^{2+}]_i$ and their transient changes, which are estimated from the difference between peak and basal levels, were detected. All experiments were performed at $25 \pm 2^{\circ}C$. Cells were superfused continuously with HEPES-buffered solution as used for confocal imaging. Cells were excited at 488 nm for FluoZin-3 and at 480 nm for fluo-3, and emissions were recorded at 520 and 515 nm, respectively. Cells showing a significant fluorescence decrease during the 5 min equilibration period were eliminated. The amplitude of Zn^{2+} or Ca^{2+} transients was stable within 10% for at least 10 min. For comparison, some cardiomyocytes were loaded with Newport Green or Calcium Green, two other previously recognized fluorophores to detect Zn^{2+} or Ca^{2+} movements (for both sparks and transients). The details of analysis of fluorescence intensity changes are given in the Supplementary material online.

2.3 Confocal imaging

Short-lived, brief and localized light emissions ('sparks') were recorded from different cardiomyocytes after 35–40 min incubation with FluoZin-3-AM or fluo-3 AM, respectively. Loaded cells were transferred to the experimental chamber (volume ~1.5 mL) mounted on the stage of a Leica TCS SP5 laser scanning microscope. Experiments were conducted on quiescent cardiomyocytes. FluoZin-3 or fluo-3 was excited at either 485 or 506 nm, and emission was collected at either 535 or 526 nm, respectively. In some experiments, recordings were obtained with HEPES-buffered solution supplemented with either $ZnCl_2$ (1, 10, or 100 μM) or zinc ionophore pyrithione (ZnPT; 1 or 10 μM), with the subsequent addition of 50 μM *N,N,N′,N′*-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) to bring the intracellular free Zn^{2+} level to zero. Experiments were performed at $25 \pm 2^{\circ}C$. An expanded Methods section is available in the Supplementary material online.

2.4 Data and statistical analysis

One-way analysis of variance (ANOVA) followed by Tukey's test was used to determine statistical significance ($P < 0.05$) (for the detail of data analysis see Suplementary material online).

3. Results

3.1 Intracellular Zn^{2+} changes in cardiomyocytes: Zn^{2+} transients and Zn^{2+} sparks

Taking advantage of the advent of new Zn^{2+}-selective and highly sensitive fluorescent dyes, we compared Zn^{2+} and Ca^{2+} changes in quiescent or electrically stimulated cardiomyocytes. Representative transitory fluorescence intensity changes of FluoZin-3- or fluo-3-loaded field-stimulated cardiomyocytes obtained by spectromicrofluorimetry are shown in *Figure 1A*. The time courses of the Zn^{2+} and Ca^{2+} transients (the averaged time to peak and half-time duration) were 236.0 ± 4.1 and 484.5 ± 18.3 ms, and 210.8 ± 7.2 and 435.3 ± 20.9 ms, respectively.

Ca^{2+} transients result from the co-ordinated release of Ca^{2+} ions from the sarcoplasmic reticulum (SR) through the ryanodine receptor activated by the Ca^{2+} influx during electrical activity. In quiescent cells, Ca^{2+} sparks represent local spontaneous SR Ca^{2+} release from clusters of ryanodine receptors, RyR2.[22] Here, for the first time, Zn^{2+} sparks are illustrated in representative line-scan images of quiescent cardiomyocytes loaded with FluoZin-3 under confocal

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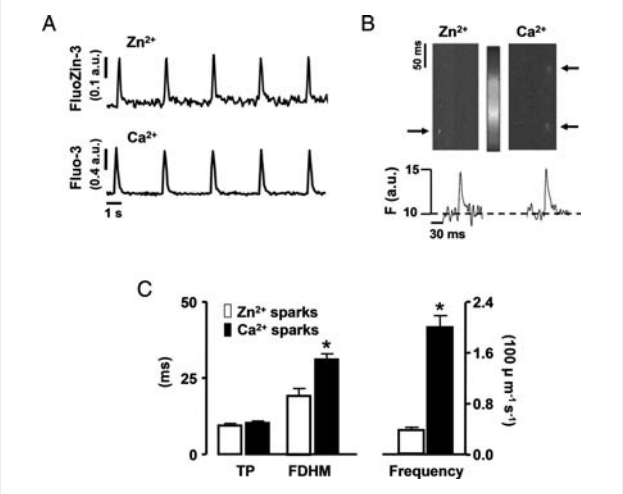


Figure 1 Intracellular Zn^{2+} changes in cardiomyocytes: Zn^{2+} transients and Zn^{2+} sparks. (*A*) Representative transitory fluorescence changes recorded by using microspectrofluorimetry in FluoZin-3- or fluo-3-loaded cardiomyocytes, with application of field-stimulation pulses at 0.2 Hz. (*B*) Representative line-scan confocal images demonstrating local fluorescence intensity changes in FluoZin-3- (left) or fluo-3-loaded quiescent cardiomyocytes (right). Line plots (below) were derived from these line-scan images at the locations indicated by arrows, showing fluorescence changes as a function of time. (*C*) Bar graphs (means ± SEM) comparing the time-to-peak amplitude (TP), full duration at half-maximum (FDHM), and frequency of fluorescence changes measured in FluoZin-3- (136 sparks from 48 cells from 13 rats) or fluo-3-loaded cardiomyocytes (629 sparks from 39 cells isolated from 12 rats). $^*P < 0.05$.

microscopy (*Figure 1B*). The Zn^{2+} sparks exhibited a rather similar time course to that of Ca^{2+} sparks recorded in fluo-3-loaded cardiomyocytes, with a relatively shorter spark duration and lower spark frequency (*Figure 1C*).

Treatment of the cells with 50 μM of the cell-permeable metal chelator TPEN resulted significant decreases in both basal FluoZin-3 fluorescence (even below the initial basal level) and the amplitude of Zn^{2+} transients, while no changes were observed in the fluorescence of fluo-3-loaded cells (*Figure 2A* and *B*). We also enforced Zn^{2+} influx using ZnPT application on FluoZin-3- or fluo-3-loaded cells in normal physiological solution (*Figure 2*). Exposure to 1 μM ZnPT induced a significant gradual increase in basal fluorescence as well as a significant increase in the amplitude of Zn^{2+} transients (approximately two-fold). The subsequent addition of TPEN decreased basal FluoZin-3 fluorescence below the initial level and suppressed the Zn^{2+} transients. Interestingly, ZnPT application significantly decreased the Ca^{2+} transients without effecting baseline fluo-3 fluorescence. Application of TPEN induced a further decrease in the fluo-3 transients (*Figure 2A* and *B*). Although we have no clear explanation, it could be proposed that TPEN influx had also been facilitated after ZnPT exposure. Then, intracellular TPEN would fully chelate Zn^{2+} and also reduced the Ca^{2+} transients, given that TPEN is a low-affinity (~300 μM) Ca^{2+} chelator.[23] Fully overlapping data were obtained when analysing the effects of TPEN and ZnPT on FluoZin-3- or fluo-3-loaded electrically stimulated cells at 37°C. Low (10 μM) EDTA, a chelator with higher affinity for Zn^{2+} than for Ca^{2+}, did not affect Zn^{2+} transients, suggesting that they do not result from Zn^{2+} entry. Furthermore, the addition of $ZnCl_2$ to the bathing solution resulted in significant decreases in the amplitude of the Zn^{2+} transients and the Ca^{2+} transients, probably as a consequence of Zn^{2+}-induced inhibition of the L-type Ca^{2+} current.[7,24] TPEN had only a weak effect on the Zn^{2+} transients and the Ca^{2+} transients following the addition of $ZnCl_2$. Basal $[Zn^{2+}]_i$ and $[Ca^{2+}]_i$ were not modified by external $ZnCl_2$ (*Figure 2A* and *B*).

Similar to the Zn^{2+} transients, the Zn^{2+} sparks were inhibited by TPEN, which caused rapid, marked decreases in FluoZin-3 intensity and frequency, whereas it did not affect fluo-3 fluorescence in quiescent cardiomyocytes (*Figure 2C* and *D*). Exposure to ZnPT induced a significant increase in the amplitude of Zn^{2+} sparks without affecting other spatiotemporal parameters of Zn^{2+} sparks, nor did it modify any Ca^{2+}-spark characteristics. On the further addition of TPEN, the amplitude of Zn^{2+} sparks decreased below the initial values, again without any alteration in the parameters of Ca^{2+} sparks. Notably, $ZnCl_2$ did not induce any change in the amplitude or frequency of Zn^{2+} sparks or Ca^{2+} sparks following a 5 min exposure period (*Figure 2C* and *D*). To confirm homogeneous loading of the cardiomyocyte with either FluoZin-3 or Newport Green, as well as its responses to either ZnPT or TPEN exposure, we also used confocal microscopy to image the full length of the cells (see Supplementary material online, *Figure S1A*). Both fluorescence loading and ionophore-induced intracellular free Zn^{2+} changes, as well as intracellular free Zn^{2+} chelation in the quiescent cell were found to be homogeneous.

In a similar series of experiments, cardiomyocytes were loaded with another fluorescent dye, either Newport Green, a highly Zn^{2+}-sensitive dye, or Calcium Green, a highly Ca^{2+}-sensitive dye. The characteristic parameters of Zn^{2+} sparks or Ca^{2+} sparks recorded in Newport Green- or Calcium Green-loaded cells (see Supplementary material online, *Figure S2A* to *D*) fully coincided with those estimated in FluoZin-3 or fluo-3-loaded cells, respectively (see also Supplementary material online, *Table S1*). The effects of TPEN and ZnPT on these parameters were also found to be comparable (see Supplementary material online, *Figure S2E* and *F*).

The recordings of Zn^{2+} sparks and Zn^{2+} transients and the effects induced by ZnPT or TPEN clearly indicate that specific intracellular Zn^{2+} changes occur in quiescent and electrically stimulated cardiomyocytes in physiological conditions, aside from the well-reported Ca^{2+} changes.

3.2 Extracellular Ca^{2+} dependency of Zn^{2+} transients and Zn^{2+} sparks

In this set of experiments, the extracellular Ca^{2+} dependency of Zn^{2+} transients and Zn^{2+} sparks was tested. Switching to a Ca^{2+}-free solution (with addition of 2 mM EGTA) abolished both the Zn^{2+} transients and the Ca^{2+} transients. Both transients recovered on re-application of the Ca^{2+}-containing solution (*Figure 3A*). Furthermore, the addition of 100 μM $CdCl_2$, known to inhibit the Ca^{2+} current, inhibited the Zn^{2+} transients as well (data not shown). In the Ca^{2+}-free solution, ZnPT could not restore Zn^{2+} transients, despite there being a clear intracellular Zn^{2+} loading (*Figure 3B*). Following exposure to ZnPT, switching to a Ca^{2+}-containing solution induced a slow but significant recovery in the FluoZin-3 transients accompanied by a rapid recovery in the fluo-3 transients. The elementary events observed in FluoZin-3- or fluo-3-loaded cardiomyocytes,

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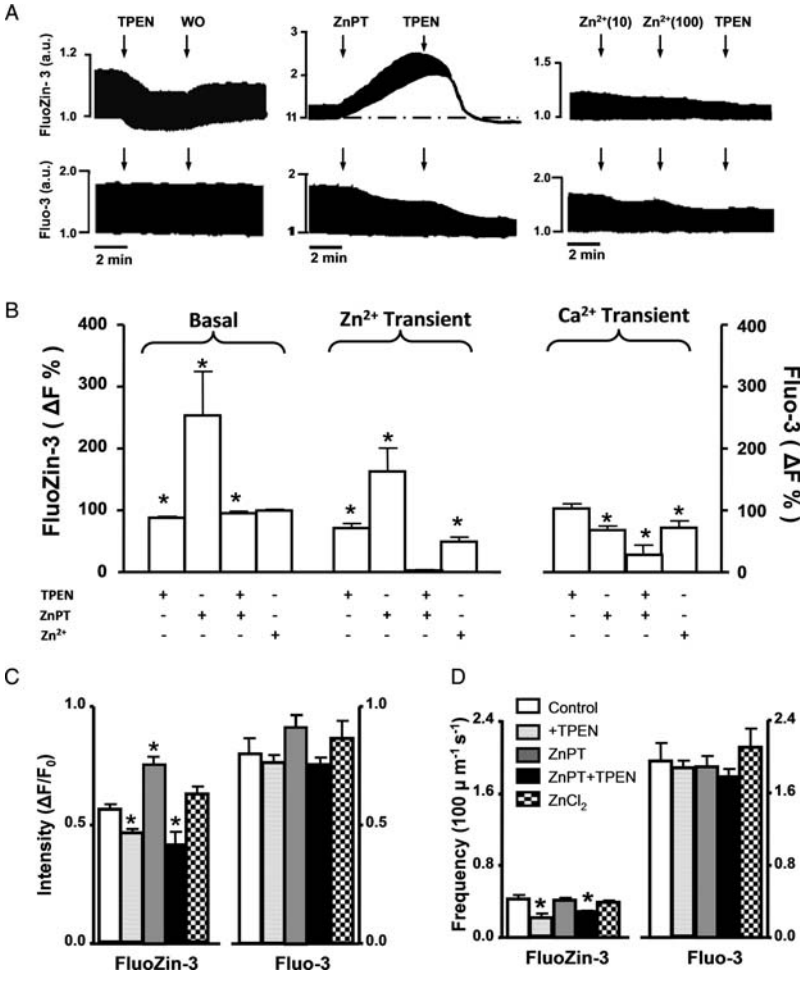


Figure 2 Effects of extracellular TPEN, ZnPT or Zn^{2+} exposure on FluoZin-3 or fluo-3 fluorescence changes in electrically field-stimulated or quiescent cardiomyocytes. (*A*) Representative transitory fluorescence changes in FluoZin-3- or fluo-3-loaded cells field stimulated at 0.2 Hz using microspectrophotometry. Cells were exposed to 50 μM TPEN for 3 min before washout (WO; left), or to 1 μM ZnPT for 5 min followed by 50 μM TPEN (middle), or to Zn^{2+} (10 μM and 100 μM, each for 5 min) followed by 50 μM TPEN (right). (*B*) Bar graphs summarizing relative effects of TPEN, ZnPT, or Zn^{2+} application on basal and transitory fluorescence changes. Values are shown as means ± SEM; 20–25 cells from four or five rats/protocols. $*P < 0.05$ vs. before application. (*C* and *D*) Bar graphs summarizing fluorescence intensities and frequencies estimated in control quiescent cardiomyocytes, and following exposures for 3 min to TPEN (50 μM), or 5 min to ZnPT (1 μM), and then 3 min after adding TPEN (50 μM), or 5 min Zn^{2+} (10 μM). Different cells were used for ZnPT or Zn^{2+} exposure protocols. $*P < 0.05$ vs. before application. Summary data (means ± SEM; >400 sparks from 15–20 cells isolated from three or four hearts).

Zn^{2+} sparks and Ca^{2+} sparks, were also both abolished in Ca^{2+}-free bathing solution and then fully recovered on switching back to Ca^{2+}-containing solution (*Figure 3C* and *D*). These data imply that intracellular Zn^{2+} changes, Zn^{2+} transients and Zn^{2+} sparks require a direct influx of Ca^{2+} and/or can arise secondarily from intracellular Ca^{2+}-dependent Ca^{2+} release.

3.3 Intracellular pools contributing to Zn^{2+} homeostasis in cardiomyocytes

We tentatively assessed the role of the SR as an intracellular Zn^{2+} pool by challenging the cardiomyocytes with ryanodine. Ryanodine (1 μM) application decreased the intensities of both Zn^{2+} transients and Ca^{2+} transients in field-stimulated cardiomyocytes without affecting basal levels (*Figure 4A* and *B*). Rather similar results were obtained in the presence of caffeine (10 mM), except for initial transitory large increases in both Zn^{2+} and Ca^{2+} basal fluorescences.

To test whether mitochondria could also be an intracellular Zn^{2+} pool, we exposed FluoZin-3- and fluo-3-loaded cells to rotenone (RT), a mitochondrial complex I inhibitor, or to carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), a mitochondrial protonophore which induces mitochondrial depolarization. Both compounds had rapid and significant inhibitory effects on the Zn^{2+}-transient amplitude with only mild initial effects on the fluo-3-loaded cells (*Figure 4A* and *B*). Similar results were observed with Newport Green- or Calcium Green-loaded cells following ryanodine or FCCP application (see Supplementary material online, *Figure S3A* and *B*).

In quiescent cells, all three compounds (ryanodine, rotenone, and FCCP) induced significant decreases in the amplitude and the

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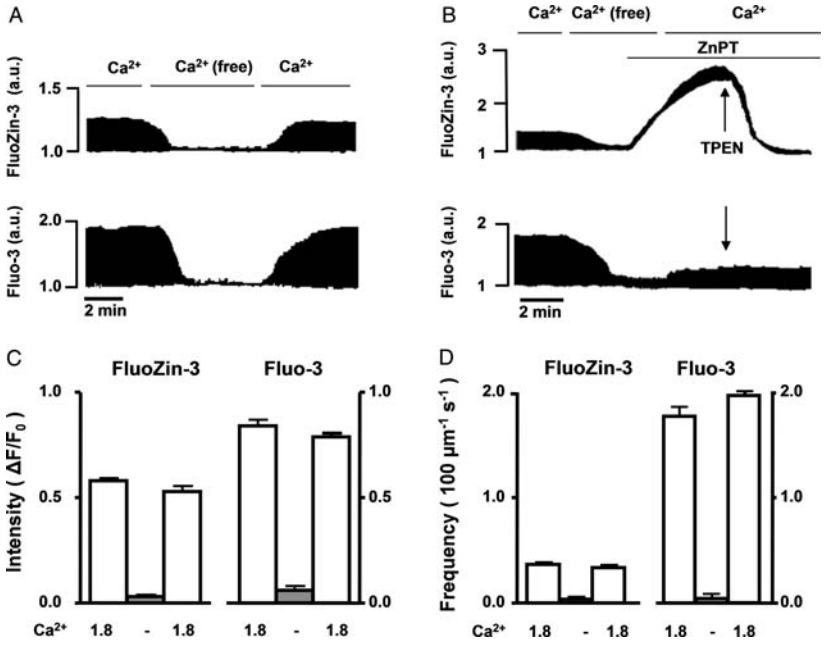


Figure 3 Extracellular Ca^{2+} dependency of Zn^{2+} transients and Zn^{2+} sparks. (*A*) Representative transitory fluorescence changes recorded in FluoZin-3- or fluo-3-loaded cells under field stimulation and superfused with a 1.8 mM Ca^{2+}-containing, or a Ca^{2+}-free (with 2 mM EGTA) HEPES-buffered solution. (*B*) Similar recordings after the addition of 1μM ZnPT and later on of 50 μM TPEN. (*C* and *D*) Spark intensities, and frequencies estimated under confocal microscopy in FluoZin-3- or fluo-3-loaded, quiescent cardiomyocytes superfused with a Ca^{2+}-containing or a Ca^{2+}-free HEPES-buffered solution. Bar graphs show pooled data (means ± SEM; >400 sparks from 10–12 cells isolated from three or four hearts).

frequency of the Zn^{2+} sparks without significantly altering the time to peak and the width duration. Suprisingly, only ryanodine showed a marked suppressing effect on the Ca^{2+} sparks (*Figure 4C* and *D*).

In further experiments, Zn^{2+} transients were analysed after increased the intracellular Ca^{2+} load by perfusing electrically stimulated cardiomyocytes with a low-Na^+-containing solution. The well-known marked increases in $[Ca^{2+}]_i$ and Ca^{2+} transients induced by reversing the Na^+–Ca^{2+} exchanger were accompanied by a significant increase in basal $[Zn^{2+}]_i$ and Zn^{2+} transients (*Figure 5A–D*). Application of low-Na^+ solution to quiescent cells often triggerred spontaneous contractions on top of the increase in basal $[Ca^{2+}]_i$ that were accompanied by a significant increase in basal $[Zn^{2+}]_i$ and the occurrence of Zn^{2+} transients (*Figure 5B*). A further application of ZnPT induced a large increase in basal $[Zn^{2+}]_i$ without significantly altering the amplitude of the Ca^{2+} transients. In the presence of ZnPT, an application of TPEN then rapidly decreased basal $[Zn^{2+}]_i$ below the initial value without altering basal $[Ca^{2+}]_i$ and Ca^{2+} transients.

To examine the physiological importance of the protein-bound Zn^{2+} pools, we induced chemical modifications of the thiol groups in the proteins by applying acidic pH to the cardiomyocytes or by inducing an oxidative stress with H_2O_2. Prior studies had found that Zn^{2+} binding to metallothioneins decreased at low pH.[25] Lowering extracellular pH to 6.0 significantly reduced the amplitude of Ca^{2+} transients without altering basal $[Ca^{2+}]_i$. It also induced a significant decrease in the amplitude of Zn^{2+} transients, which was accompanied by a marked increase in basal $[Zn^{2+}]_i$. This pH-dependent effect was more pronounced in the Zn^{2+} transients than in the Ca^{2+} transients (*Figure 5E* and *F*). The pH-dependent effect on basal $[Zn^{2+}]_i$ but not on basal $[Ca^{2+}]_i$ was rapidly antagonized with TPEN. The effect of low pH on Zn^{2+} homeostasis was also fully reversible on switching pH back to a physiological level (not shown).

Using fura-2, which shows affinity for both Ca^{2+} and Zn^{2+}, we previously estimated that the oxidative agents hypochlorous acid or selenite induced up to a 30-fold increase in basal $[Zn^{2+}]_i$, with little effect on resting $[Ca^{2+}]_I$, as expected.[3] In this experimental series, we used another strong oxidant agent, H_2O_2, at a relatively low concentration on FluoZin-3- or fluo-3-loaded cardiomyocytes. Application of 100 μM H_2O_2 induced significant increases in basal $[Ca^{2+}]_i$ and $[Zn^{2+}]_i$, which occurred with rather similar time courses (*Figure 6*). Of note, both Zn^{2+} and Ca^{2+} transients were significantly increased. The sulfydryl-reducing agent dithiothreitol (DTT) rapidly and fully restored the initial fluorescence levels of both cations. Furthermore, TPEN antagonized the H_2O_2-induced Zn^{2+} changes without affecting the Ca^{2+} changes.

4. Discussion

Given the multiple roles of Zn^{2+} in various cell types and the recent development of selective fluorophores, we investigated the changes in Zn^{2+} homeostasis in cardiomyocytes during the excitation–contraction cycle. Using the recently developed Zn^{2+}-sensitive fluorescent dye, FluoZin-3, we demonstrated that marked changes in intracellular Zn^{2+} occur during electrical activity as well as in quiescent cardiomyocytes, where Zn^{2+} sparks are revealed. Zinc movements in cardiomyocytes are controlled by changes in intracellular free Ca^{2+} and result for the most part from ionic exchange on intracellular binding sites, which is highly sensitive to the cell oxidative state and pH.

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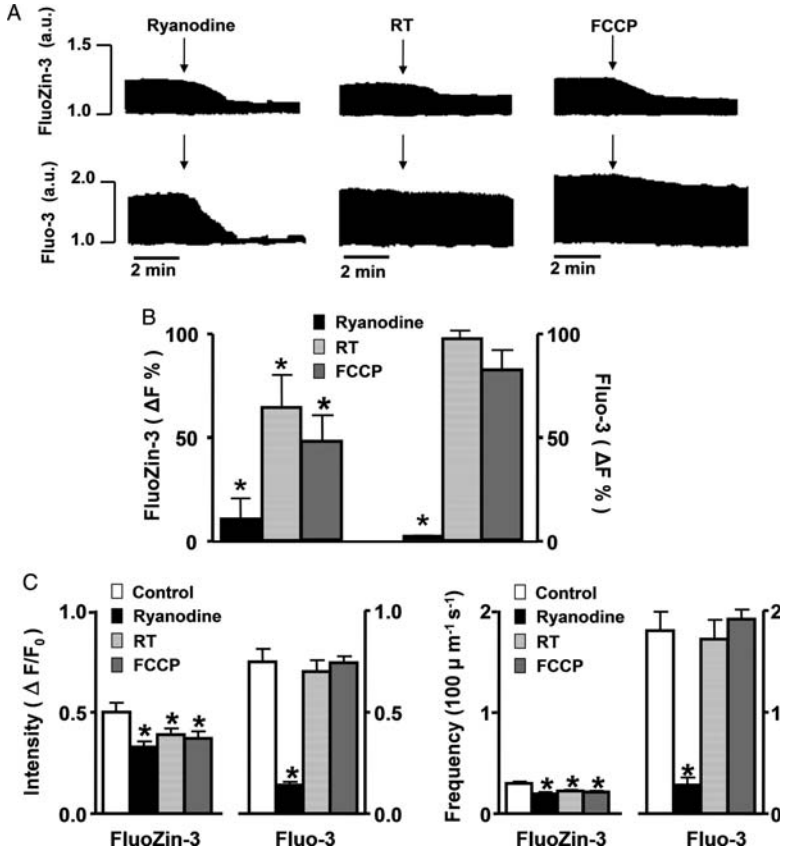


Figure 4 Assessing intracellular Zn^{2+} stores in cardiomyocytes: involvement of sarcoplasmic reticulum and mitochondria. (*A*) Representative transitory fluorescence changes for Zn^{2+} and Ca^{2+} recorded in FluoZin-3- or fluo-3-loaded, field-stimulated cardiomyocytes exposed to 1 μM ryanodine (left), 1 μM rotenone (RT; middle), or 1 μM FCCP (right) at the indicated times. (*B*) Pooled data displaying relative changes in fluorescence (ΔF) induced in the above conditions. $n = 15-20$ cells from five or six rats. $^*P < 0.05$ vs. before application. (*C* and *D*) Spark intensities, and frequencies estimated under confocal microscopy in FluoZin-3- or fluo-3-loaded, quiescent cardiomyocytes superfused with 1 μM ryanodine, 1 μM RT, or 1 μM FCCP. Bars are means ± SEM; >400 sparks from 10–12 cells from three or four hearts.

In this study, we used a new Zn^{2+} indicator, FluoZin-3, which is (i) sensitive to nanomolar levels of Zn^{2+} with a prodigious quantum yield; (ii) highly selective for Zn^{2+} and insensitive to Ca^{2+} and Mg^{2+}; (iii) stable in the presence of oxidants; and (iv) effectively able to measure dynamic changes in Zn^{2+}, and demonstrates a homogeneous staining pattern in resting cells.[19,26,27] Here, we report the occurrence of Zn^{2+} transients in field-stimulated cells which have very similar kinetics to those of Ca^{2+} transients. Additionally, in quiescent cardiomyocytes, we demonstrated, for the first time, the existence of Zn^{2+} sparks, which have similar kinetics to those of Ca^{2+}. To reinforce that changes in FluoZin-3 fluorescence are in fact dependent on $[Zn^{2+}]_i$, we applied ZnPT (a Zn^{2+} ionophore) and observed a marked increase in the events recorded with FluoZin-3-loaded cells, while application of TPEN (a membrane-permeant Zn^{2+}-specific chelator) induced a marked decrease in these events without any significant change in those recorded with fluo-3-loaded cells. Similar results were also obtained using Newport Green-loaded cells. The short-term (less than seconds) intracellular changes in Zn^{2+} were hardly dependent of Zn^{2+} entry, since the addition of $ZnCl_2$ to the bathing solution rather inhibited both Ca^{2+} and Zn^{2+} transients as a consequence of inhibition of the Ca^{2+} current by internal or external Zn^{2+},[7,24] while the addition of a low concentration of EDTA to chelate extracellular Zn^{2+} had no effect. These results extend previous observations of variations in basal $[Zn^{2+}]_i$ and Zn^{2+} transients in electrically stimulated cardiomyocytes using fura-2, a fluorophore sensitive to both Ca^{2+} and Zn^{2+} cations,[3,7] or FluoZin-3.[28] In the present work, Zn^{2+} transients and Zn^{2+} sparks were also observed with similar characteristics using another Zn^{2+}-selective fluorecent dye, Newport Green, despite its less favourable kinetics.

The similarities of the kinetics of Zn^{2+} and Ca^{2+} movements led us to check the role of Ca^{2+} in $[Zn^{2+}]_i$ homeostasis. In neurons, intracellular Zn^{2+} release that occurs over several minutes with application of KCl or glutamate depends on Ca^{2+} entry.[26,29] Also, in mast cells, extracellular stimulation-induced Zn^{2+} release from the perinuclear area leading to Zn^{2+} waves requires Ca^{2+} entry.[18] Here, in cardiomyocytes, most of the rapid (less than seconds) changes in Zn^{2+} were suppressed by removing external Ca^{2+} or by applying a Ca^{2+}-current blocker. This implies that, following Ca^{2+} entry or Ca^{2+}-induced Ca^{2+} release, the elevated $[Ca^{2+}]_i$ triggers Zn^{2+} release from intracellular sources. Considering that calsequestrin, a Zn^{2+}-binding protein that resides in the SR, can bind up to 200 mol Zn^{2+}/mol independently of binding up to 50 mol Ca^{2+}/mol,[30] we first checked the effects of ryanodine and caffeine. Both compounds triggered similar inhibition of Zn^{2+} and Ca^{2+} transients.

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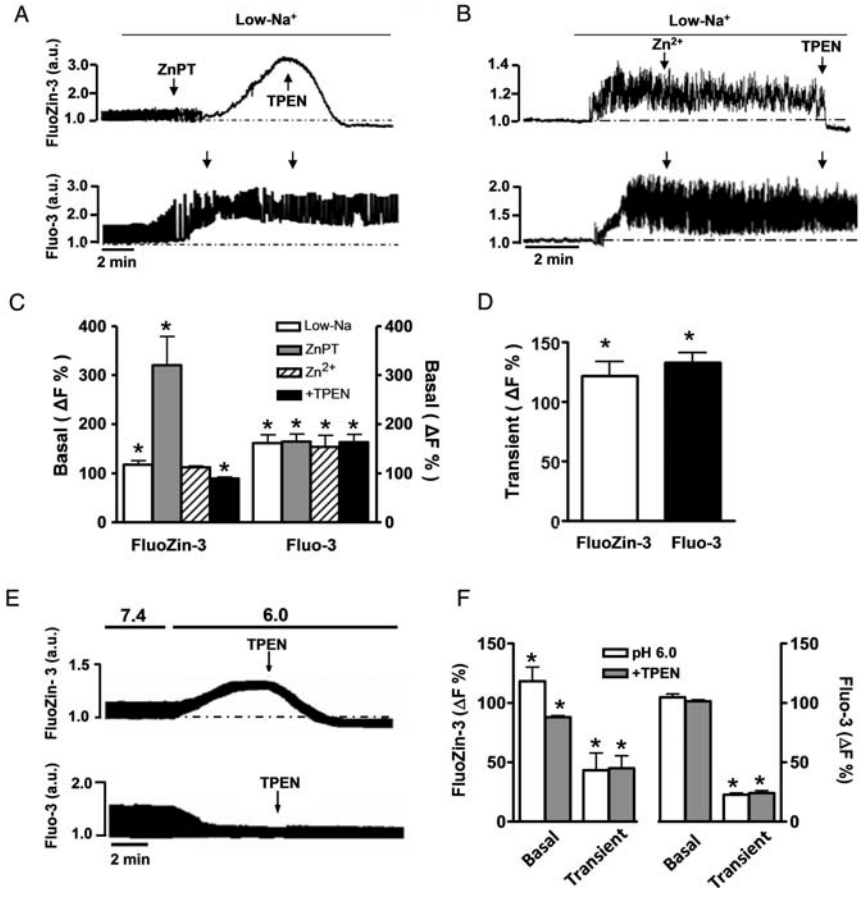


Figure 5 Assessing intracellular Zn^{2+} stores: responses to extracellular low Na^+ and extracellular acidification. (*A*) Representative recordings of Zn^{2+} and Ca^{2+} transients in FluoZin-3- or fluo-3-loaded, field-stimulated cardiomyocytes exposed to a 10 mM Na^+-containing solution (Low-Na^+), then with added 1 μM ZnPT and later on with 50 μM TPEN. (*B*) Representative recordings of Zn^{2+} and Ca^{2+} transients in FluoZin-3- or fluo-3-loaded, initially quiescent cardiomyocytes exposed to a 10 mM Na^+-containing solution, with later addition of 10 μM $ZnCl_2$ (Zn^{2+}) and then 50 μM TPEN. (*C* and *D*) Bar graphs summarizing the effects of low Na^+, followed by TPEN, ZnPT, or Zn^{2+} exposure on basal (*C*) or transitory fluorescence changes (*D*) in either FluoZin-3- or fluo-3-loaded cells (means ± SEM).*$P < 0.05$ vs. before application; 7–12 cardiomyocytes isolated from three or four hearts/protocols. (*E*) Representative recordings of Zn^{2+} and Ca^{2+} transients in FluoZin-3- or fluo-3-loaded, field-stimulated cardiomyocytes exposed to an acidic HEPES-buffered solution (pH 6.0), then exposed to 50 μM TPEN. Note that in both sets of experimental conditions, TPEN restored the basal Zn^{2+} level without affecting either basal or transient Ca^{2+}. (*F*) Bar graph summarizing the effects of acidic pH followed by TPEN exposure on basal or transitory fluorescent changes in either FluoZin-3- or fluo-3-loaded cells. Values are shown as means ± SEM; *$P < 0.05$ vs. before application; 7–12 cardiomyocytes from three hearts/protocols.

In addition, overloading the cardiomyocytes with Ca^{2+} by reversing the Na^+–Ca^{2+} exchanger with an external low-Na^+ solution induced increases in basal levels and transients of both cations.

In the present experiments, mitochondrial activity inhibitors have shown little short-term effects (of the order of minutes) on Ca^{2+} transients and Ca^{2+} sparks, while they inhibited intracellular Zn^{2+} changes significantly. In neurons, it was proposed that the Ca^{2+}-dependent Zn^{2+} release originates largely from mitochondria.[29] The Ca^{2+} dependency of intracellular Zn^{2+} mobilization by glutamate was also attributed to the generation of ROS arising from both cytosolic and mitochondrial sources.[26] In cardiomyocytes, mitochondria occupy 30–40% of the cellular volume and constitute the major source of intracellular ROS production. Up to 1–2% of the electrons in the electron transfer chain leak to molecular oxygen to form superoxide anion and, subsequently, H_2O_2.[31,32] Mitochondria-related excessive ROS production has been implicated in the pathogenesis of many cardiovascular diseases, including ischaemia–reperfusion injury. Furthermore, cardiac mitochondria produce NO that mobilizes intracellular Zn^{2+} via cGMP–protein kinase G signalling.[17] Mitochondrial NO production increases with increasing Ca^{2+} concentration that may result from Ca^{2+} uptake by the Ca^{2+} uniporter.[33] Collapsing mitochondrial membrane potential with the protonophore FCCP or blocking the respiratory chain with rotenone inhibits mitochondrial NO production.[34] Such results could account for our observations that FCCP and rotenone reduced intracellular Zn^{2+} changes compared with control conditions. Further work is needed to elucidate how much these mechanisms could also contribute to the mitochondrial inhibitor-resistent Zn^{2+} release.

Excess Zn^{2+} in cells is buffered by binding to cysteine, histidine, or glutamate residue of thousands of metalloproteins, including metallothioneins. Based on their variable redox state and Zn^{2+} binding, the properties of metallothioneins are consistent with that of

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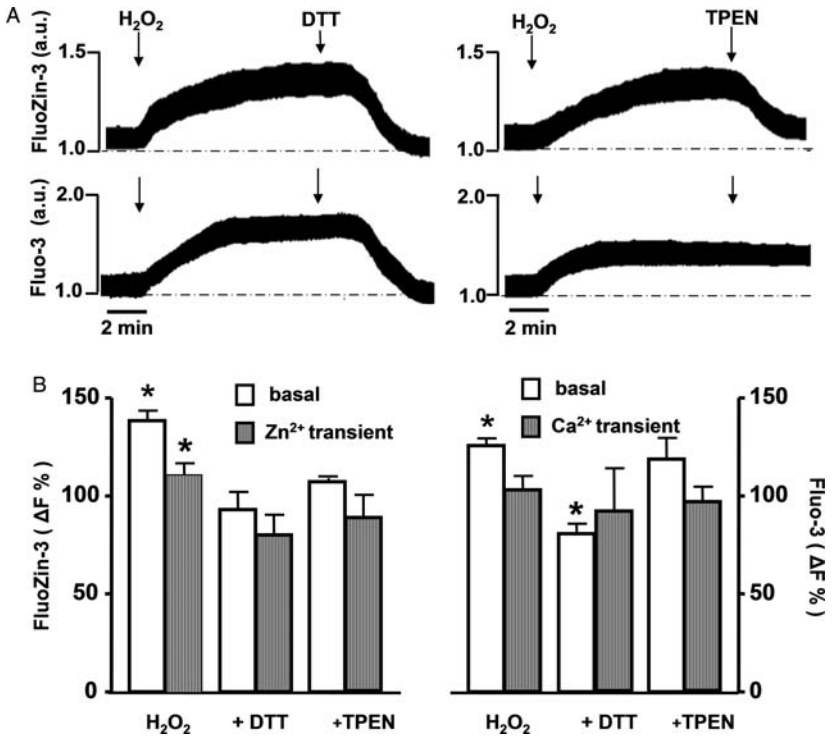


Figure 6 Assessing intracellular Zn^{2+} stores: responses to oxidant and antioxidant. (*A*) Representative recordings of Zn^{2+} and Ca^{2+} transients in FluoZin-3- or fluo-3-loaded, field-stimulated cardiomyocytes exposed to 100 μM H_2O_2 for 10 min, followed by either 500 μM dithiothreitol (DTT; left), or 50 μM TPEN (right). (*B*) Bar graphs displaying the effects of H_2O_2, DTT, or TPEN exposure on basal or transitory fluorescent changes in either FluoZin-3- or fluo-3-loaded cells. Values are shown as means ± SEM; $^*P < 0.05$ vs. before application; 15–20 cardiomyocytes from four or five hearts/protocols.

a redox protein and have a regulatory role in Zn^{2+} metabolism.[35] Zn^{2+} release from metallothioneins occurs in conditions of oxidative stress. ROS species promote NO generation from inducible nitric oxide synthase, which in turn promotes the release of Zn^{2+} from the Zn^{2+}–metallothionein complex.[16,35,36] In line with these observations, we showed that H_2O_2 application induces an increase in Zn^{2+} transients elicited with unaltered Ca^{2+} transients. Furthermore, the equilibrium of free vs. chelated Zn^{2+} is sensitive to environmental factors that include acidosis, such as during ischaemia. Acidic pH appears to rapidly destabilize Zn^{2+}–thiol binding to release free Zn^{2+},[34] consequently reducing the available amount of Ca^{2+}-dependent releasable Zn^{2+}. However, similar to oxidizing conditions, acidosis appears to increase the efficiency of Ca^{2+} to release Zn^{2+}.

Furthermore, taking into account that metallothioneins as well as other metalloproteins could also act as a thermodynamic sink,[37] part of the Ca^{2+}-dependent Zn^{2+} release could be independent of ROS production. Thus, it cannot be fully excluded that Zn^{2+} release was triggered by intracellular increases in Ca^{2+} that displaced Zn^{2+} from binding sites on various metalloproteins. In this situation, the redox satus or the pH would modulate the available amount of exchangeable Zn^{2+} and the Ca^{2+} efficiency.

Besides metallothioneins, carnosine (β-alanine-histidine) another low-molecular-weight compound that forms complexes with various heavy metals, including Zn^{2+}, has antiradical effects and antioxidative activity.[38,39] However, carnosine effects would be more marked in skeletal tissues, where it is much more abundant than in the cardiac muscle.[40]

To conclude, based on ischaemia–reperfusion studies in cardiomyocytes, it was proposed that there might be a critical threshold beyond which mitochondria become dysfunctional and damaged as a consequence of ROS exceeding and/or depleted antioxidant defence.[41] Weber and colleagues[4] further suggested that an optimal intracellular Zn^{2+}/Ca^{2+} ratio in cardiomyocytes and mitochondria must be preserved to combat oxidative stress. They reported that increased $[Ca^{2+}]_i$ and mitochondrial $[Ca^{2+}]$ were coupled to the induction of oxidative stress, while antioxidant effects result from the rise in $[Zn^{2+}]_i$ and mitochondrial $[Zn^{2+}]$ accompanied by a simultaneous activation of metal response element transcription factor-1, and its induction of such antioxidants as metallothionein-1 and glutathione peroxidase. This is also reminiscent of the Ca^{2+} overloading and oxidative stress observed in the hearts of rodents with hereditary muscular dystrophy, which is also accompanied by increased $[Zn^{2+}]_i$.[40] Our present results suggest that the Ca^{2+}-induced Zn^{2+} release allows for an optimal ratio even on a very short time scale. Moreover, it is interesting to note that during short-term oxidizing conditions, as used here, the Ca^{2+}-induced Zn^{2+} release, which can demonstrate pro-antioxidant properties, is favoured. For how long and to what level the increase in $[Zn^{2+}]_i$ would be beneficial or become detrimental remain to be investigated.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Conflict of interest: none declared.

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References

1. Murakami M, Hirano T. Intracellular zinc homeostasis and zinc signaling. *Cancer Sci* 2008;**99**:1515–1522.
2. Vallee BL, Falchuk KH. The biochemical basis of zinc physiology. *Physiol Rev* 1993;**73**: 79–118.
3. Turan B, Fliss H, Désilets M. Oxidants increase intracellular free Zn^{2+} concentration in rabbit ventricular myocytes. *Am J Physiol* 1997;**272**:H2095–H2106.
4. Kamalov GPA, Ahokas RA, Zhao W, Zhao T, Shahbaz AU, Johnson PL *et al*. Coupled calcium and zinc dyshomeostasis and oxidative stress in cardiac myocytes and mitochondria of rats with chronic aldosteronism. *J Cardiovasc Pharmacol* 2009;**53**:414–423.
5. Andreini C, Banci L, Bertini I, Rosato A. Zinc through the three domains of life. *J Proteome Res* 2006;**5**:3173–3178.
6. Eide DJ. The SLC39 family of metal ion transporters. *Pflugers Arch* 2004;**447**: 796–800.
7. Atar D, Backx PH, Appel MM, Gao WD, Marban E. Excitation-transcription coupling mediated by zinc influx through voltage-dependent calcium channels. *J Biol Chem* 1995;**270**:2473–2477.
8. Palmiter RD, Huang L. Efflux and compartmentalization of zinc by members of the SLC30 family of solute carriers. *Pflugers Arch* 2004;**447**:744–751.
9. Ohana E, Segal D, Palty R, Ton-That D, Moran A, Sensi SL *et al*. A sodium zinc exchange mechanism is mediating extrusion of zinc in mammalian cells. *J Biol Chem* 2004;**279**:4278–4284.
10. Sekler I, Sensi SL, Hershfinkel M, Silverman WF. Mechanism and regulation of cellular zinc transport. *Mol Med* 2007;**13**:337–343.
11. Maret W. The function of zinc metallothionein: a link between cellular zinc and redox state. *J Nutr* 2000;**130**:1455S–1458S.
12. Lengyel I, Fieuw-Makaroff S, Hall AL, Sim AT, Rostas JA, Dunkley PR. Modulation of the phosphorylation and activity of calcium/calmodulin-dependent protein kinase II by zinc. *J Neurochem* 2000;**75**:594–605.
13. Brautigan DL, Pinault FM. Activation of membrane protein-tyrosine phosphatase involving cAMP- and Ca^{2+}/phospholipid-dependent protein kinases. *Proc Natl Acad Sci USA* 1991;**88**:6696–6700.
14. Winegar BD, Lansman JB. Voltage-dependent block by zinc of single calcium channels in mouse myotubes. *J Physiol* 1990;**425**:563–578.
15. Sensi SL, Jeng JM. Rethinking the excitotoxic ionic milieu: the emerging role of Zn^{2+} in ischemic neuronal injury. *Curr Mol Med* 2004;**4**:87–111.
16. St Croix CM, Wasserloos KJ, Dineley KE, Reynolds IJ, Levitan ES, Pitt BR. Nitric oxide-induced changes in intracellular zinc homeostasis are mediated by metallothionein/thionein. *Am J Physiol Lung Cell Mol Physiol* 2002;**282**:L185–L192.
17. Jang Y, Wang H, Xi J, Mueller RA, Norfleet EA, Xu Z. NO mobilizes intracellular Zn^{2+} via cGMP/PKG signalling pathway and prevents mitochondrial oxidant damage in cardiomyocytes. *Cardiovasc Res* 2007;**75**:426–433.
18. Yamasaki S, Sakata-Sogawa K, Hasegawa A, Suzuki T, Kabu K, Sato E *et al*. Zinc is a novel intracellular second messenger. *J Cell Biol* 2007;**177**:637–645.
19. Gee KR, Zhou ZL, Ton-That D, Sensi SL, Weiss JH. Measuring zinc in living cells. A new generation of sensitive and selective fluorescent probes. *Cell Calcium* 2002; **31**:245–251.
20. Zhao J, Bertoglio BA, Devinney MJ Jr, Dineley KE, Kay AR. The interaction of biological and noxious transition metals with the zinc probes FluoZin-3 and Newport Green. *Anal Biochem* 2009;**384**:34–41.
21. 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**:3082–3088.
22. Cheng H, Lederer WJ, Cannell MB. Calcium sparks: elementary events underlying excitation-contraction coupling in heart muscle. *Science* 1993;**262**:740–744.
23. Jung C, Zima AV, Szentesi P, Jona I, Blatter LA, Niggli E. Ca^{2+} release from the sarcoplasmic reticulum activated by the low affinity Ca^{2+} chelator TPEN in ventricular cardiomyocytes. *Cell Calcium* 2007;**41**:187–194.
24. Turan B. Zinc-induced changes in ionic currents of cardiomyocytes. *Biol Trace Elem Res* 2003;**94**:49–60.
25. Jiang LJ, Vasák M, Vallee BL, Maret W. Zinc transfer potentials of the α- and β-clusters of metallothionein are affected by domain interactions in the whole molecule. *Proc Natl Acad Sci USA* 2000;**97**:2503–2508.
26. Dineley KE, Devinney MJ 2nd, Zeak JA, Rintoul GL, Reynolds IJ. Glutamate mobilizes [Zn^{2+}] through Ca^{2+}-dependent reactive oxygen species accumulation. *J Neurochem* 2008;**106**:2184–2193.
27. Devinney MJ 2nd, Reynolds IJ, Dineley KE. Simultaneous detection of intracellular free calcium and zinc using fura-2FF and FluoZin-3. *Cell Calcium* 2005;**37**:225–232.
28. Palmer BM, Vogt S, Chen Z, Lachapelle RR, Lewinter MM. Intracellular distributions of essential elements in cardiomyocytes. *J Struct Biol* 2006;**155**:12–21.
29. Sensi SL, Ton-That D, Weiss JH. Mitochondrial sequestration and Ca^{2+}-dependent release of cytosolic Zn^{2+} loads in cortical neurons. *Neurobiol Dis* 2002;**10**:100–108.
30. Baksh S, Spamer C, Oikawa K, McCubbin WD, Heilmann C, Kay CM *et al*. Zn^{2+} binding to cardiac calsequestrin. *Biochem Biophys Res Commun* 1995;**209**:310–315.
31. Dedkova EN, Blatter LA. Mitochondrial Ca^{2+} and the heart. *Cell Calcium* 2008;**44**: 77–91.
32. Chance B, Sies H, Boveris A. Hydroperoxide metabolism in mammalian organs. *Physiol Rev* 1979;**59**:527–605.
33. Duchen MR. Mitochondria and calcium: from cell signalling to cell death. *J Physiol* 2000;**529**:57–68.
34. Maret W. Molecular aspects of human cellular zinc homeostasis: redox control of zinc potentials and zinc signals. *Biometals* 2009;**22**:149–157.
35. Spahl DU, Berendji-Grün D, Suschek CV, Kolb-Bachofen V, Kröncke KD. Regulation of zinc homeostasis by inducible NO synthase-derived NO: nuclear metallothionein translocation and intranuclear Zn^{2+} release. *Proc Natl Acad Sci USA* 2003;**100**: 13952–13957.
36. Khatai L, Goessler W, Lorencova H, Zangger K. Modulation of nitric oxide-mediated metal release from metallothionein by the redox state of glutathione *in vitro*. *Eur J Biochem* 2004;**271**:2408–2416.
37. Rana U, Kothinti R, Meeusen J, Tabatabai NM, Krezoski S, Petering DH. Zinc binding ligands and cellular zinc trafficking: apo-metallothionein, glutathione, TPEN, proteomic zinc, and Zn-Sp1. *J Inorg Biochem* 2008;**102**:489–499.
38. Boldyrev A, Song R, Lawrence D, Carpenter DO. Carnosine protects against excitotoxic cell death independently of effects on reactive oxygen species. *Neuroscience* 1999;**94**:571–577.
39. Stuerenburg HJ, Kunze K. Concentrations of free carnosine (a putative membrane-protective antioxidant) in human muscle biopsies and rat muscles. *Arch Gerontol Geriatr* 1999;**29**:107–113.
40. Crawford AJ, Bhattacharya SK. Excessive intracellular zinc accumulation in cardiac and skeletal muscles of dystrophic hamsters. *Exp Neurol* 1987;**95**:265–276.
41. Aon MA, Cortassa S, O'Rourke B. Redox-optimized ROS balance: a unifying hypothesis. *Biochim Biophys Acta* 2010;**1797**:865–877.

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