



## Regulatory volume decrease in cardiomyocytes is modulated by calcium influx and reactive oxygen species

Diego Rojas-Rivera<sup>a</sup>, Jessica Díaz-Elizondo<sup>a</sup>, Valentina Parra<sup>a</sup>, Daniela Salas<sup>a</sup>, Ariel Contreras<sup>a</sup>, Barbra Toro<sup>a</sup>, Mario Chiong<sup>a,b</sup>, Claudio Olea-Azar<sup>c</sup>, Sergio Lavandero<sup>a,b,d,\*</sup>

<sup>a</sup>Centro FONDAP Estudios Moleculares de la Célula, Departamentos de, Santiago 838-0492, Chile

<sup>b</sup>Bioquímica y Biología Molecular, Santiago 838-0492, Chile

<sup>c</sup>Química Inorgánica y Analítica, Facultad de Ciencias Químicas y Farmacéuticas, Santiago 838-0492, Chile

<sup>d</sup>Instituto Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago 838-0492, Chile

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### ABSTRACT

**We investigated the role of Ca<sup>2+</sup> in generating reactive oxygen species (ROS) induced by hyposmotic stress (Hypo) and its relationship to regulatory volume decrease (RVD) in cardiomyocytes. Hypo-induced increases in cytoplasmic and mitochondrial Ca<sup>2+</sup>. Nifedipine (Nife) inhibited both Hypo-induced Ca<sup>2+</sup> and ROS increases. Overexpression of catalase (CAT) induced RVD and a decrease in Hypo-induced blebs. Nife prevented CAT-dependent RVD activation. These results show a dual role of Hypo-induced Ca<sup>2+</sup> influx in the control of cardiomyocyte viability. Hypo-induced an intracellular Ca<sup>2+</sup> increase which activated RVD and inhibited necrotic blebbing thus favoring cell survival, while simultaneously increasing ROS generation, which in turn inhibited RVD and induced necrosis.**

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### 1. Introduction

Preservation of a constant volume is critical for eukaryotic cells. Cell volume is disturbed by exposure to anisotonic environments [1,2]. Cells adapt to hyposmotic stress (Hypo) by a variety of mechanisms [3]. Restitution of cell volume after cell swelling in mammalian cells is achieved by the loss of solutes (K<sup>+</sup>, Cl<sup>-</sup>, and organic osmolytes) and the subsequent osmotically driven efflux of water. This process is known as regulatory volume decrease (RVD) [2,4,5]. The ability of cells to resist osmotic swelling or shrinkage by cell volume regulation parallels their resistance to necrosis or apoptosis after osmotic shock [2,6].

*Abbreviations:* Ad, adenovirus; CAT, catalase; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; DCF-DA, 2',7'-dichlorofluorescein diacetate; DMPO, 5,5-dimethyl-1-pyrroline N-oxide; ESR, electron spin resonance; HO·, hydroxyl radical; Hypo, hyposmotic stress; Iono, ionomycin; Iso, isosmotic; LacZ, β-galactosidase; LDH, lactic dehydrogenase; MOI, multiplicity of infection; Nife, nifedipine; O<sub>2</sub><sup>-</sup>, superoxide; ONOO, peroxynitrite; ROI, region of interest; ROS, reactive oxygen species; RuRed, ruthenium red; RVD, regulatory volume decrease; Vera, verapamil

\* Corresponding author. Address: Centro FONDAP Estudios Moleculares de la Célula, Departamentos de, Santiago 838-0492, Chile. Fax: +56 2 678 2912.

E-mail address: [slavander@uchile.cl](mailto:slavander@uchile.cl) (S. Lavandero).

Isolated cardiac cells are not normally exposed to changes in extracellular osmolarity [2,5]. However, in some pathological conditions, such as ischemia and reperfusion, diabetic shock, myocardial infarction and septic shock, such cells are exposed to hyposmotic as well as hyperosmotic stress [5,7]. Alterations in cell volume may seriously affect cardiac function [8]. Cell swelling represents a major threat to the heart both acutely by promoting the development of fatal arrhythmias [9] and chronically by increasing infarct size occasioned by accelerated necrosis [10]. The pro-arrhythmic effect of swelling arises mainly from alterations in electrophysiological properties [5].

Exposure of cultured rat cardiomyocytes to Hypo stimulates a rapid swelling without any compensatory RVD. Hypo increased reactive oxygen species (ROS) production, mainly involving NADPH oxidase. Adenoviral expression of catalase (CAT) inhibits Hypo-dependent hydroxyl radical (HO·) production, induces a RVD mechanism and prevents cell death [11].

Ca<sup>2+</sup> has also been associated with cell volume regulation mechanisms [12]. In Ehrlich cells, incubation with the Ca<sup>2+</sup> ionophore A23187 causes cell shrinkage under isosmotic conditions, underlining the importance of intracellular [Ca<sup>2+</sup>] in volume regulation [13]. In lymphocytes, Ehrlich cells, and Chinese hamster ovary

cells, RVD does not require extracellular  $\text{Ca}^{2+}$ , but depletion of intracellular  $\text{Ca}^{2+}$  abolishes the activation of  $\text{K}^+$  channels by swelling [12]. Participation of  $\text{Ca}^{2+}$  in cardiomyocyte volume regulation is controversial.

The participation of  $\text{Ca}^{2+}$  in ROS production induced by Hypo and its relationship with modulation of RVD have not been investigated. Here we show a dual role of Hypo-induced  $\text{Ca}^{2+}$  influx in the control of cardiomyocyte viability. Hypo increases  $\text{Ca}^{2+}$ -dependent ROS generation which inhibited RVD and triggers necrosis. Simultaneously cytoplasmic  $\text{Ca}^{2+}$  increase activates RVD and inhibits necrotic blebbing thus favoring cell survival.

## 2. Materials and methods

### 2.1. Animals

Rats were bred in the Animal Breeding Facility from the Faculty of Chemical and Pharmaceutical Sciences, University of Chile (Santiago, Chile). This investigation conforms to the “Guide for the care and use of laboratory animals” published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985) and it was approved by our institutional Review Board and Ethics Committee.

### 2.2. Culture of rat cardiomyocytes

Neonatal rat cardiomyocytes were prepared from hearts of 1–3-day-old Sprague–Dawley rats as described previously [14]. Cultured cardiomyocytes were identified using an anti  $\beta$ -myosin heavy chain antibody and cell cultures were  $\geq 95\%$  pure [14]. Hyposmotic culture media ( $248 \pm 5$  and  $202 \pm 5$  mosmol (kg water) $^{-1}$ ) were made by diluting culture media with distilled water (15% and 30% dilution, respectively). When cell volume was measured, experiments were also performed using an isotonic solution containing 95 mM NaCl, 5 mM KCl, 0.5 mM  $\text{MgCl}_2$ , 1.3 mM  $\text{CaCl}_2$ , 10 mM HEPES, pH 7.4, and sucrose was added to yield a final osmolarity of 310 mosmol (kg water) $^{-1}$ .

### 2.3. Adenovirus and inhibitors

Adenovirus catalase (AdCAT) was transduced at a multiplicity of infection (MOI) of 300. As control, an  $\beta$ -galactosidase (AdLacZ) construct was used. Cells were used after incubation for 24 h at 37 °C in DMEM/M199. Cardiomyocytes were preincubated with nifedipine (Nife) (10  $\mu\text{M}$ ) for 30 min before addition of hyposmotic culture media. AdCAT was provided by the Gene Transfer Vector Core (University of Iowa, USA) [15].

### 2.4. Measurement of cytoplasmic and mitochondrial calcium

To determine cytoplasmic and mitochondrial  $\text{Ca}^{2+}$  levels, images were obtained from cultured cardiomyocytes preloaded with fluo3-AM or rhod2-AM using an inverted confocal microscope (Carl Zeiss LSM-5, Pascal 5 Axiovert 200 microscope). Cardiomyocytes were washed three times with  $\text{Ca}^{2+}$ -containing resting media (Krebs buffer) to remove DMEM/M199 culture medium, and loaded with 5.4  $\mu\text{M}$  fluo3-AM or 200 nM rhod2-AM for 30 min at room temperature. After loading, cells were washed either with the same buffer or with  $\text{Ca}^{2+}$ -free resting media (145 mM NaCl, 5 mM KCl, 1 mM EGTA, 1 mM  $\text{MgCl}_2$ , 10 mM HEPES-Na, 5.6 mM glucose, pH 7.4) and used within 2 h. Cells containing coverslips were mounted in a 1-ml capacity chamber and placed in the microscope for fluorescence measurements after excitation with a laser line of 488-nm for fluo3-AM or 543-nm for rhod2-AM. Hyposmotic medium was fast (1 s) changed in the chamber. The fluorescent

images were collected every 0.4–2.0 s for fast signals and analyzed frame by frame with Image J software (NIH, Bethesda, MD). For intracellular calcium measurements, a manual contour of the whole cell was generated while for mitochondrial calcium, an optical region of interest (ROI) on perinuclear mitochondrion were analyzed. To quantify fluorescence, the summed pixel intensity was calculated from the section delimited by the contour or the mitochondrial ROI. Intracellular  $\text{Ca}^{2+}$  levels were expressed as relative total fluorescence [ $\Delta F/F_0$ : ratio of fluorescence difference, stimulated-basal ( $F_i - F_0$ ), to basal value ( $F_0$ )] as a function of time. The fluorescence intensity increases proportionally with intracellular  $\text{Ca}^{2+}$  [16]. Digital image processing was performed as previously described [16]. Under our experimental conditions (0–100 s), no photobleaching was observed. In order to compare  $\text{Ca}^{2+}$  signal induced by Hypo, two additional controls were done. The maximal  $\text{Ca}^{2+}$  response was attained with KCl (80 mM) or ionomycin (Iono) (1  $\mu\text{M}$ ), reaching 20–25-fold increases whereas Hypo triggers twofold increase in cytosolic  $\text{Ca}^{2+}$  levels (Supplementary Fig. S1). Although fluo-3 dye was compartmentalized in the nucleus, any kinetic difference between cytosolic and nuclear  $\text{Ca}^{2+}$  was observed.

### 2.5. Measurement of ROS production

Cardiomyocytes exposed to hyposmotic solutions were treated 10 min before cell lysis with 10  $\mu\text{M}$  2',7'-dichlorofluorescein diacetate (DCF-DA) in complete darkness. Cells were lysed with 100 mM NaOH and kept on ice avoiding light exposure. Fluorescence was determined in cell extracts (excitation: 490 nm, emission: 525 nm). The lysates were always keeping on ice until measuring. Relative fluorescence was determined in all our experimental setting. The basal fluorescence was measured using cell lysates obtained from non DCF-DA preloaded cardiac myocytes (autofluorescence). The maximal fluorescence was determined using cell lysates obtained from DCF-DA preloaded and hydrogen peroxide (1 mM) pretreated cardiac myocytes. Arbitrary units of fluorescence were corrected for protein content determined by Bradford assay.

Additionally, cardiomyocytes were exposed to hyposmotic solutions in the presence of 200 mM 5,5-dimethyl-1-pyrroline N-oxide (DMPO). Cells were incubated for 30 min at 37 °C in complete darkness, lysed with 0.5 ml DME/M199 4:1 containing Triton X-100 (0.8% v/v), 200 mM DMPO and incubated for 10 min at 37 °C. In the presence of DMPO,  $\text{HO}^\bullet$  were trapped as the more stable spin adducts, DMPO-OH. Electron spin resonance (ESR) spectra were recorded in the X-band (9.85 GHz) using a Bruker ECS 106 spectrometer with a rectangular cavity and 50 kHz field modulation. The hyperfine splitting constants were estimated to be accurate within 0.05 G [17].

### 2.6. Measurement of cell volume

Modifications in cell water volume were assessed in single cardiomyocytes by measuring changes in concentration of calcein, an intracellularly trapped fluorescent dye, as previously described [11,18].

### 2.7. Statistical analysis

Data are given as mean  $\pm$  S.E.M. of a number of independent experiments ( $n$ ) as indicated or as the mean of representative experiments performed on at least three separate occasions with similar outcome. Data were analyzed by ANOVA and comparisons were performed using a protected Tukey's test. A value of  $P < 0.05$  was set as the limit of statistical significance.

### 3. Results

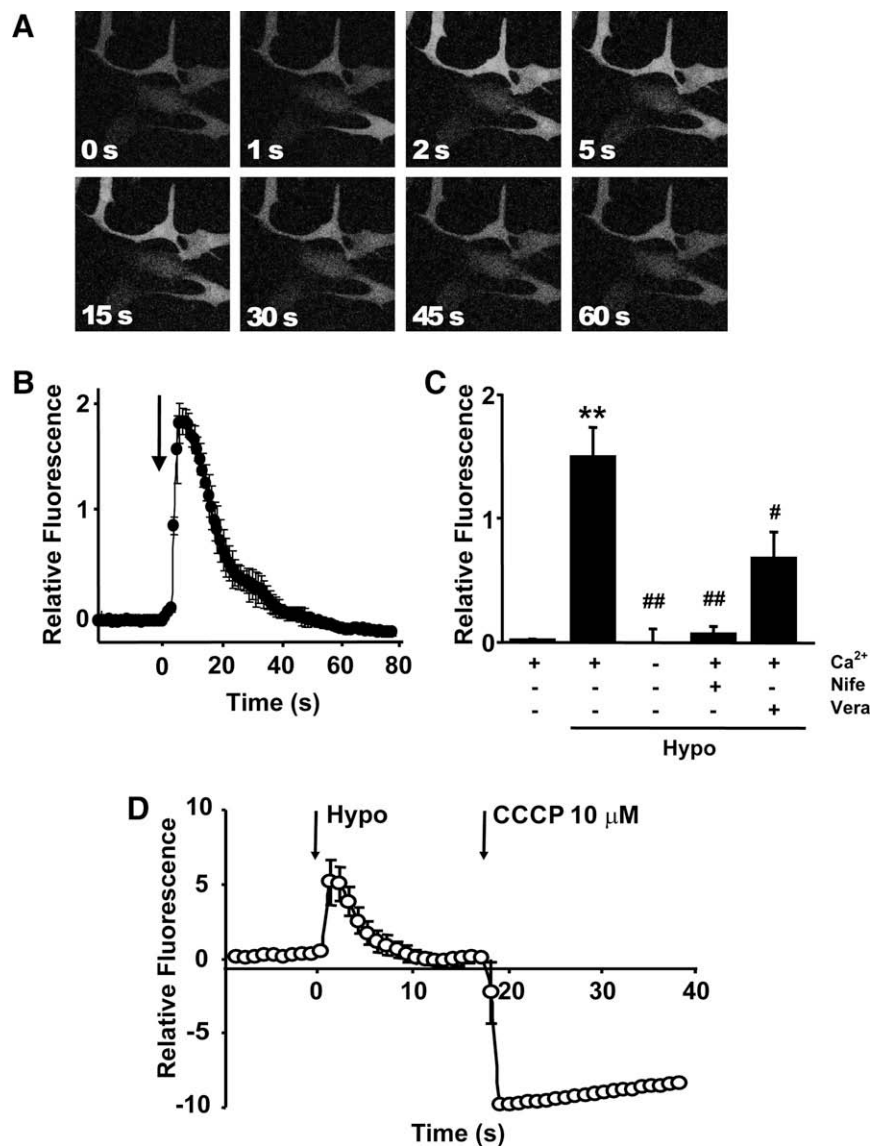
#### 3.1. Hyposmotic stress increases $[Ca^{2+}]_i$ in cultured cardiomyocytes

Cardiomyocytes were preloaded with fluo3-AM, and the  $[Ca^{2+}]_i$  was monitored by relative fluorescence ( $\Delta F/F_0$ ) of single cells for each series of images. Cells in  $Ca^{2+}$ -containing medium were exposed to Hypo (202 mosmol  $(kg\ water)^{-1}$ ). A sequence of fluorescence images showing a fast and transitory  $[Ca^{2+}]_i$  increase is shown in Fig. 1A. Eighty-five percent of the cells (30 cells of 35 from five different cultures) responded as shown in Fig. 1A. Whole cell fluorescence analysis showed a strong and transient  $Ca^{2+}$  signal (20–40 s duration) that begun at 1 or 5 s and peaked at 3–9 s after exposure to Hypo (Fig. 1B). ROI analysis showed no differences between nuclear and cytoplasmic  $Ca^{2+}$  signal (Supplementary Fig. S2). When cardiomyocytes were exposed to the same Hypo

condition in a  $Ca^{2+}$ -free resting medium, no fluorescence increase was detected (Fig. 1C). Pretreatment with 10  $\mu M$  Nife or 50  $\mu M$  verapamil (Vera) for 30 min suppressed the rise of fluorescence induced by Hypo (Fig. 1C).

An increase in cytoplasmic  $Ca^{2+}$  can induce  $Ca^{2+}$  uptake by the mitochondria. In order to detect mitochondrial  $Ca^{2+}$  increases associated to the cytoplasmic  $Ca^{2+}$  signal, cardiomyocytes were preloaded with rhod2-AM. Hypo-induced a mitochondrial  $Ca^{2+}$  increase of 10 s duration that peaked at 2–3 s after hyposmotic exposure (Fig. 1D). Mitochondrial uncoupling using the protonophore carbonyl cyanide 3-chlorophenylhydrazone (CCCP, 10  $\mu M$ ) induced a drastically reduction of mitochondrial  $Ca^{2+}$  (Fig. 1D).

These results suggest that Hypo induces cytoplasmic and mitochondrial  $Ca^{2+}$  transients arising from an external  $Ca^{2+}$  influx through an L-type  $Ca^{2+}$  channel.



**Fig. 1.** Hyposmotic stress induces cytoplasmic and mitochondrial calcium increases. Cultured rat cardiomyocytes were preloaded with fluo3-AM (cytoplasmic) or with rhod2-AM (mitochondrial) for 30 min at room temperature and afterward hyposmotic stress was induced (202 mosm  $(kg\ water)^{-1}$ ). (A) Series of temporal images showing fluo3 fluorescence changes determined by confocal microscopy in cultured cardiomyocytes expose to hyposmotic stress in  $Ca^{2+}$ -containing medium. (B) Temporal relative fluorescence change of fluo3 was calculated from fluorescence images in A. Black arrow indicates the time when the hyposmotic stress was induced. (C). Rates of rise of fluo3 relative fluorescence changes in cardiomyocytes exposed to isosmotic (290 mosm  $(kg\ water)^{-1}$ ) and hyposmotic stress (Hypo) in presence and absence of extracellular calcium and 10  $\mu M$  nifedipine (Nife) or 50  $\mu M$  verapamil (Vera). (D) Mitochondrial  $Ca^{2+}$  determined as rhod2 relative fluorescence by confocal microscopy in cultured cardiomyocytes. Arrows indicate the time when the hyposmotic stress (Hypo) and 10  $\mu M$  CCCP were added. Data are mean  $\pm$  S.E.M. and the figures are representatives of 3–4 independent experiments. \*\* $P < 0.01$  vs. control, ## $P < 0.01$  and # $P < 0.05$  vs. Hypo- $Ca^{2+}$ .

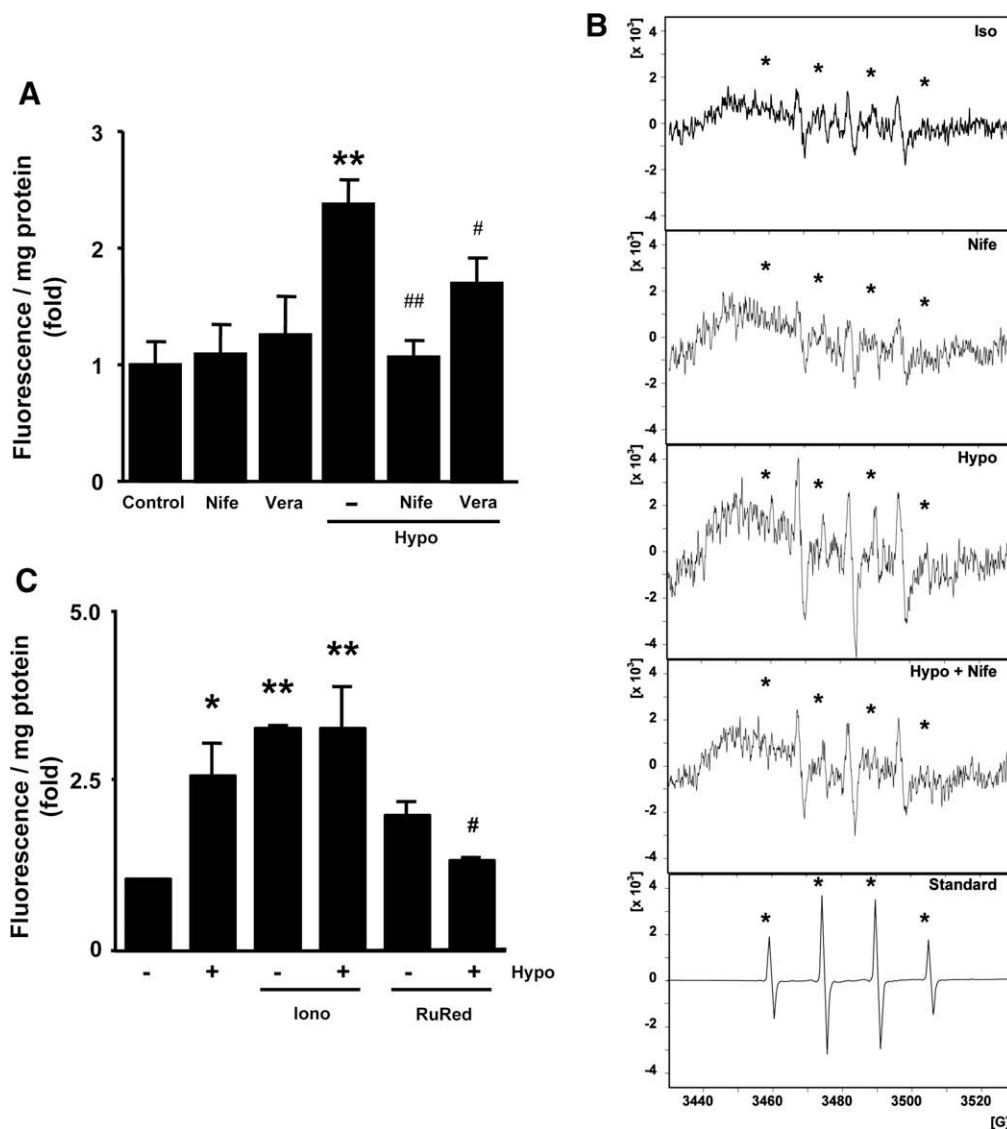
### 3.2. Hyposmotic stress stimulates ROS production by $Ca^{2+}$

Hypo stimulates ROS production in cardiomyocytes [11]. To evaluate the participation of  $Ca^{2+}$  influx in Hypo-induced ROS production, cells were preincubated with 10  $\mu$ M Nife for 30 min and ROS production was determined after exposure to Hypo. Hypo increased ROS  $2.3 \pm 0.3$ -fold with respect to controls ( $P < 0.01$ ) (Fig. 2A). When cells were pretreated with Nife, Hypo-dependent ROS generation was significantly decreased to  $1.1 \pm 0.3$ -fold respect to control ( $P < 0.01$  vs. hypotonic). However Nife addition 5 s after Hypo did not significantly decrease Hypo-dependent ROS formation ( $2.1 \pm 0.3$  over control). This data suggest that Nife does not have an important antioxidant effect in our model. Fig. 2A shows that Vera induced a partial but significant decrease in ROS ( $1.8 \pm 0.3$ -fold respect to control,  $P < 0.05$  vs. Hypo). These results show that Nife and Vera blocked Hypo-induced ROS produc-

tion in cardiomyocytes, suggesting that  $Ca^{2+}$  influx is necessary for ROS formation.

Exposure of cardiomyocytes to Hypo in the presence of the ESR spin trap DMPO, generated a four-line (1:2:2:1) ESR spectrum with  $AN = AH = 14.9$  G (Fig. 2B). These ESR spectral characteristics were consistent with the formation of a relatively stable nitroxide- $HO\cdot$  spin adduct [19]. When cells were preincubated with 10  $\mu$ M Nife, the 1:2:2:1 ESR spectrum induced by Hypo was prevented (Fig. 2B). These results suggest that  $Ca^{2+}$  influx through the L-type  $Ca^{2+}$  channel was responsible for ROS production, in particular  $HO\cdot$ .

To confirm  $Ca^{2+}$  increase-dependent ROS generation, cardiomyocytes were treated with Iono. This  $Ca^{2+}$  ionophore increased ROS formation in a similar level to that observed with Hypo (Fig. 2C). No further increase in ROS levels was observed by hypotonic exposure of Iono-treated cardiomyocytes (Fig. 2C), suggest-



**Fig. 2.** Calcium influx induces ROS generation in cultured cardiomyocytes exposed to hypotonic stress. (A) Cells were preincubated with 10  $\mu$ M DCF-DA and afterwards hypotonic stress was induced for 15 min in the presence or absence of 10  $\mu$ M nifedipine (Nife) or 50  $\mu$ M verapamil (Vera). Cell lysates were collected and their fluorescence was determined as indicated in Section 2. (B) Cardiomyocytes were preloaded with 10  $\mu$ M nifedipine (Nife), except the control, and incubated in isosmotic medium (Iso) or under hypotonic stress (Hypo) condition in the presence of 200 mM DMPO for 30 min. Cells were lysed and extracts were analyzed by electron spin resonance (ESR) using DMPO as "spin trap". Asterisks indicate the position of the signals corresponding to the DMPO-OH adduct. (C) Cells were preincubated with 10  $\mu$ M DCF-DA and afterwards hypotonic stress was induced for 15 min in the presence or absence of 1  $\mu$ M ionomycin (Iono) or 10  $\mu$ M ruthenium red (RuRed). Cell lysates were collected and their fluorescence was determined as indicated in Section 2. Data are mean  $\pm$  S.E.M. and the figures are representatives of three independent experiments. \*\* $P < 0.01$  and \* $P < 0.05$  vs. control, ## $P < 0.01$  and # $P < 0.05$  vs. Hypo.

ing that the ROS production induced by Iono and Hypo was not additive.

In order to evaluate the participation of mitochondrial  $\text{Ca}^{2+}$  increase induced by Hypo in ROS formation, cardiomyocytes were pretreated with ruthenium red (RuRed), an inhibitor of mitochondrial uniporter, and then exposed to Hypo. Preincubation with RuRed completely block Hypo-induced ROS generation (Fig. 2C). This result suggests that ROS formation was dependent on mitochondrial  $\text{Ca}^{2+}$  increase induced by Hypo.

### 3.3. Calcium influx is necessary for RVD in cardiomyocytes that overexpress catalase

Cardiomyocytes did not display measurable RVD after 15 min exposure to Hypo [11]. Increased in CAT activity did, however, completely restore RVD in hyposmotically-stressed cardiomyocytes (Fig. 3). CAT-dependent RVD was completely blocked by pretreatment with Nife (Fig. 3). Taken together these results suggest that RVD can be restored by CAT overexpression and that  $\text{Ca}^{2+}$  influx induced by Hypo is important for the regulation of this RVD.

### 3.4. Hyposmotic stress-induced cell blebbing is modulated by calcium and ROS

Hypo induces caspase-independent cell death, with cell blebbing and release of lactic dehydrogenase (LDH) [11]. These phenomena together are consistent with necrotic cell death. To investigate whether cell blebbing is modulated by both  $\text{Ca}^{2+}$  influx

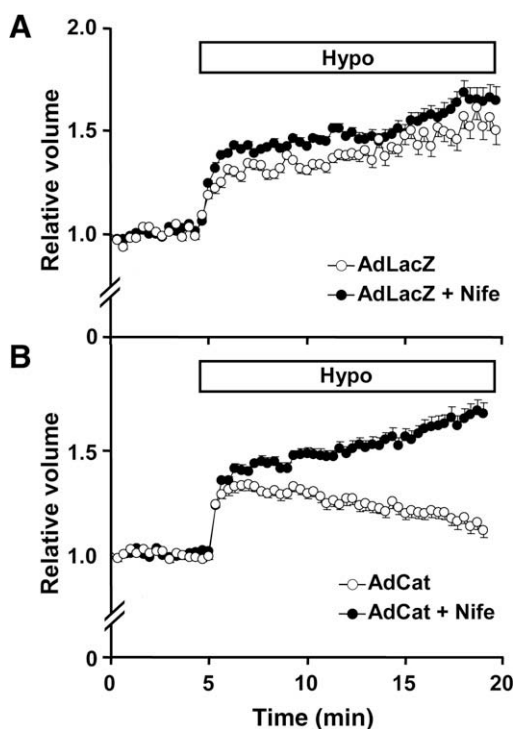
and ROS generation induced by Hypo, cells were preloaded with calcein-AM, exposed to Hypo and cell blebbing was evaluated by confocal microscopy, as is shown in Fig. 4A. Hypo-induced necrotic bleb-like structures in  $70 \pm 13\%$  of cardiomyocytes. Overexpression of CAT protected cells from cell death induced by Hypo exposure, when compared with those overexpressing LacZ as control. When cardiomyocytes overexpressing CAT were preincubated with  $10 \mu\text{M}$  Nife, they lost the ability to make RVD (Fig 3B) and present an increase in cell blebbing (Fig. 4B). These results suggest that protection by overexpression of CAT in exposed to hyposmotically-stressed cells needs  $\text{Ca}^{2+}$  influx through the L-type  $\text{Ca}^{2+}$  channel. Moreover, the increasing of Hypo-induced cell blebbing after Nife pretreatment suggests that the  $\text{Ca}^{2+}$  influx could be involved in the inhibition of such blebbing.

## 4. Discussion

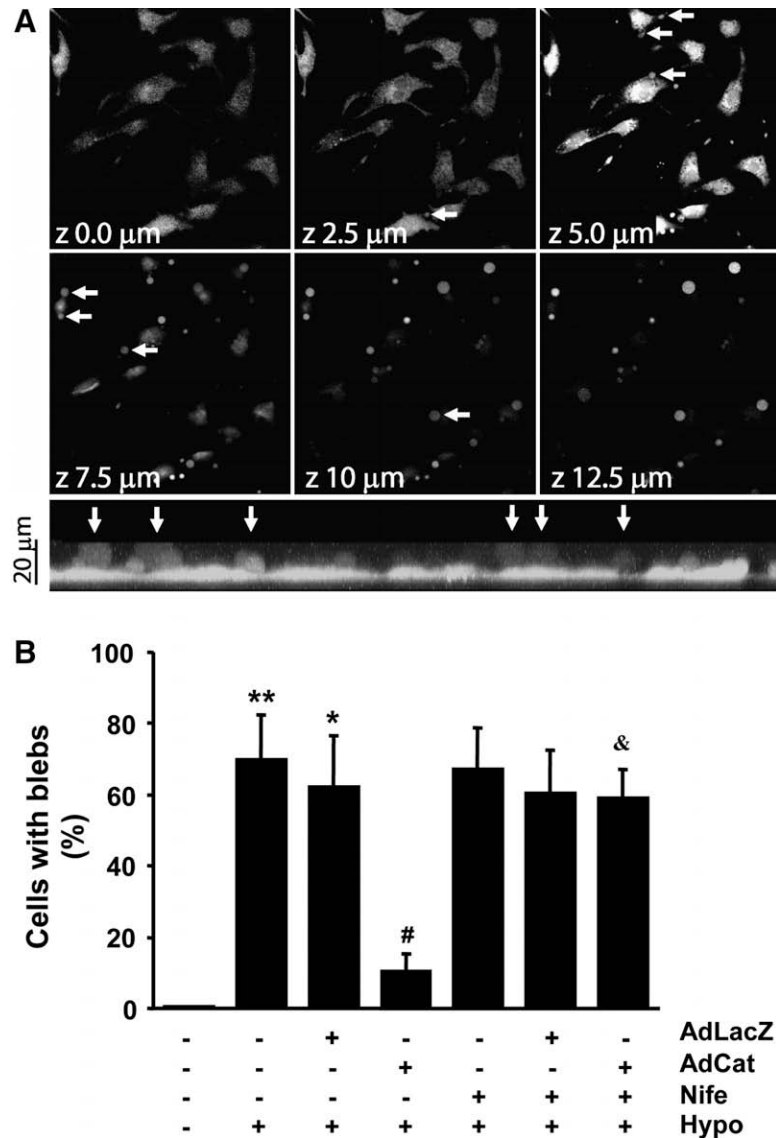
In different cell types, osmolarity changes induce a  $[\text{Ca}^{2+}]_i$  signal that is critical for the regulation of both ion and osmolyte flows involved in the RVD process. Our results have shown that Hypo produced a fast increase in  $[\text{Ca}^{2+}]_i$ , which was important in ROS generation, RVD regulation and inhibition of necrotic bleb formation.

The RVD process depends on different factors such as intra- and extra-cellular  $[\text{Ca}^{2+}]$ , activation of the MAPK pathway and the presence and activity of ionic channels [2,20–22]. We have previously shown that Hypo induces an increase in ROS in cultured cardiomyocytes, this increase being associated with an RVD inhibition and a decrease in cell viability [11]. RVD was, however, observed in these cells when CAT was overexpressed, suggesting a relationship between ROS and RVD. Here we found that, in such cells, RVD did not occur when the L-type  $\text{Ca}^{2+}$  channel was blocked with Nife, suggesting the involvement of a  $\text{Ca}^{2+}$  influx in the RVD-regulatory action of CAT. In epithelial cells, buffering with  $\text{Ca}^{2+}$  chelators prevents RVD [23]. A normal RVD response can, however, occur in Ehrlich cells [24] as well as in salivary acinar cells [25] and lymphocytes [26] without any rise in intracellular  $[\text{Ca}^{2+}]_i$ . Taouil et al. have described that Hypo induces a fast and transient  $[\text{Ca}^{2+}]_i$  increase in cardiomyocytes [27]. This phenomenon was abolished in calcium-free hypotonic media, in the presence of diltiazem and by membrane pre-depolarization by exposure to high  $[\text{K}^+]$  media, suggesting an involvement of L-type voltage-activated calcium channels [27]. Takeushi et al. showed that, in guinea pig ventricular myocytes, intracellular  $\text{Ca}^{2+}$  accumulation via the window current of the L-type  $\text{Ca}^{2+}$  channel causes rapid swelling through the  $\text{Ca}^{2+}$ -activated background cation current-triggered membrane depolarization [28]. Here we have demonstrated that Hypo-induced a strong and transient  $\text{Ca}^{2+}$  signal. ROI analysis showed no differences between nuclear and cytoplasmic  $\text{Ca}^{2+}$  signals (Supplementary Fig. S2). These transient  $[\text{Ca}^{2+}]_i$  increases were inhibited by calcium-free hypotonic media, Nife and Vera, suggesting also the involvement of a L-type  $\text{Ca}^{2+}$  channel. In guinea pig ventricular myocytes, hyposmotic swelling reduces the L-type  $\text{Ca}^{2+}$  current [29]. However, both osmotic and hydrostatic inflation increased the L-type  $\text{Ca}^{2+}$  current in rabbit atrial and sino-atrial myocytes [30]. This difference may be because of variation between the species chosen or the methodological procedure used.

$\text{Ca}^{2+}$  [27] and ROS [11] are two important factors in the regulation and integration of cellular function, and crosstalk between them has been described [31–33]. During hypoxia, ROS production by mitochondria and NAD(P)H oxidase modulate the L-type  $\text{Ca}^{2+}$  channel activity in cardiac myocytes [34]. We observed, however, that overexpression of CAT did not modify the increase of  $\text{Ca}^{2+}$  induced by Hypo, but inhibited the ROS increase [11]. Moreover, inhibition of L-type  $\text{Ca}^{2+}$  channel with Nife and Vera decreased



**Fig. 3.** Calcium influx and ROS affects regulated volume decrease (RVD) in cultured cardiomyocytes. Cells were transduced with (panel A) adenovirus  $\beta$ -galactosidase (AdLacZ) or (panel B) adenovirus catalase (AdCat) and incubated for 24 h. Transduced cells were preloaded with  $5 \mu\text{M}$  calcein-AM for 10 min at  $37^\circ\text{C}$  in the presence or absence of  $10 \mu\text{M}$  nifedipine (Nife), washed with isotonic medium and exposed to hyposmotic stress (Hypo) in the presence or absence of  $10 \mu\text{M}$  nifedipine. Changes in the intracellular calcein concentration were determined by confocal microscopy. Relative volume was calculated for each point as described in Section 2. Data are mean  $\pm$  S.E.M. and the figures are representatives of 3–4 independent experiments.



**Fig. 4.** Hyposmotic stress-induced cell blebbing is modulated by calcium and ROS. Cultured rat cardiomyocytes were preloaded with 5  $\mu\text{M}$  calcein-AM for 10 min at 37  $^{\circ}\text{C}$  and then exposed to hypotonic stress (Hypo). Cells were transduced with adenovirus catalase (AdCat) or adenovirus  $\beta$ -galactosidase (AdLacZ) and incubated with 10  $\mu\text{M}$  nifedipine (Nife) for 30 min as described in Section 2. (A) Blebs induced by hypotonic medium are shown as representative images of confocal z slice section of 0.5  $\mu\text{m}$  each, from the bottom to the apical zone of the sample. Blebs induced by 30 min of hypotonic stress are indicated by arrows. A 20  $\mu\text{m}$  projection in X-axis is also shown. (B) Quantification of cells with blebs after 20 min treatment with hypotonic medium (Hypo, 202 mosm (kg water) $^{-1}$ ). Data are mean  $\pm$  S.E.M. and the figures are representatives of 3–4 independent experiments. \*\* $P < 0.01$  and \* $P < 0.05$  vs. control; # $P < 0.05$  vs. AdLacZ-Hypo; & $P < 0.05$  vs. AdCat-Hypo.

the Hypo-induced ROS. Treatment of cultured cardiomyocytes with Iono, a  $\text{Ca}^{2+}$  ionophore, increased ROS levels in a similar extent to that observed with Hypo. Moreover, treatment of Iono-pre-incubated cardiomyocytes with Hypo did not induce an additional ROS increase. These results suggest that  $\text{Ca}^{2+}$  is the responsible for ROS formation triggered by Hypo.

The decrease of ROS production by Nife could also result from dihydropyridines having antioxidant activity [35]. Nife did not, however, decrease ROS basal levels, suggesting that this drug has a minor effect on ROS decrease in our system. Vera, a  $\text{Ca}^{2+}$  channel blocker without antioxidant activity [36], also decreased Hypo-induced ROS formation. Taken together, these data suggest that  $\text{Ca}^{2+}$  influx through the L-type  $\text{Ca}^{2+}$  channel was responsible for ROS production in hypototically-stressed cardiomyocytes.

We have previously shown that BAPTA-AM, rotenone and apocynin inhibit the Hypo-dependent ROS increase, suggesting that ROS are produced by both the mitochondrial respiratory chain and NAD(P)H oxidase [11]. Here we demonstrated that this process

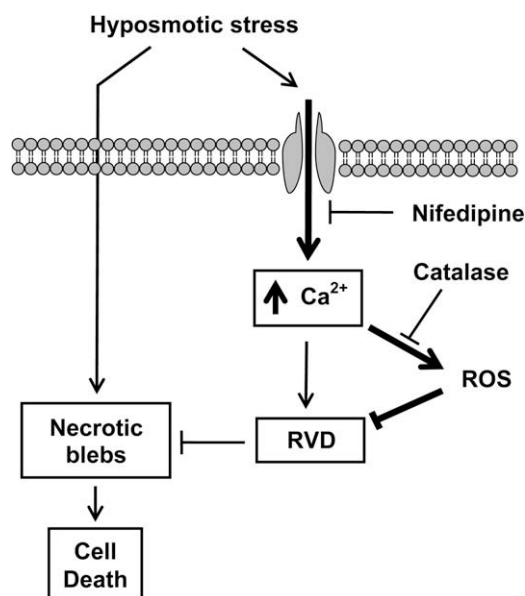
takes place through a  $[\text{Ca}^{2+}]_i$ -dependent mechanism. Our results showed that Hypo-induced increases in both cytoplasmic and mitochondrial  $\text{Ca}^{2+}$ . Inhibition of mitochondrial  $\text{Ca}^{2+}$  uniporter with RuRed completely suppressed ROS increase induced by Hypo. These results suggest that an increase in mitochondrial  $\text{Ca}^{2+}$  was responsible for ROS formation. It has been reported that an increase in  $[\text{Ca}^{2+}]_i$  induces both a decrease in mitochondrial potential and an increase in the ROS level [37]. High concentrations of mitochondrial  $\text{Ca}^{2+}$  trigger opening of the mitochondrial permeability transition pores and enhance ROS production [33].

In addition to the regulation of mitochondrial ROS production,  $\text{Ca}^{2+}$  regulates multiple extra-mitochondrial ROS generating enzymes. Cell-surface NAD(P)H oxidase is the most important enzyme in the generation of ROS [38,39]. NAD(P)H oxidase is present in variety of cells including cardiomyocytes [33,34,39]. NADPH oxidase 5 (NOX5), a homolog of the gp91phox subunit of the phagocyte NADPH oxidase, generates large amounts of superoxide ( $\text{O}_2^-$ ) when exposed to increases in  $[\text{Ca}^{2+}]_i$ ; this is attributable

to a conformation change of NOX5 induced by  $\text{Ca}^{2+}$  [40]. Schliess et al. studied the effect of  $[\text{Ca}^{2+}]_i$  increase on ROS production in astrocytes exposed to Hypo [41]. They demonstrated that Hypo induces protein tyrosine nitration consequent upon an NMDA receptor-mediated increase in  $[\text{Ca}^{2+}]_i$ , which leads to a calmodulin-dependent generation of reactive oxygen- and nitrogen intermediates. Nitric oxide (NO) can recombine with the  $\text{O}_2^-$  anion to form the very potent nitrating species peroxynitrite (ONOO) [41]. We have shown that Hypo increased  $\text{HO}^\cdot$  and that overexpression of CAT inhibited this increase correlating with RVD recovery [11]. These data suggest that ROS directly or through the formation of other reactive species such as  $\text{ONOO}^-$  could be inhibiting the RVD through redox modification of a membrane channel.

Bleb formation is a characteristic of both apoptotic and necrotic cell death. Apoptotic blebs increase their volume and then shrink as the regulatory volume increase process progresses. Necrotic blebs are produced before cell membrane lysis, grow indefinitely and do not shrink as do the apoptotic blebs; this behavior has been associated with enhancement of cell volume and to necrotic volume increase [42]. We have previously described formation of necrotic blebs in cardiomyocytes exposed to Hypo, and that this was inhibited by overexpression of CAT [11]. Here we observed that influx of  $\text{Ca}^{2+}$  through an L-type calcium channel is important for the inhibition of blebbing in cardiomyocytes overexpressing CAT. These data suggest that ROS production is important in the formation of necrotic blebs, contributing to cardiomyocyte death by inhibition of RVD.

In summary, our results suggest that exposure of cardiomyocytes to Hypo triggers a  $\text{Ca}^{2+}$  influx through an L-type  $\text{Ca}^{2+}$  channel. The increase of intracellular  $\text{Ca}^{2+}$  activates RVD, but simultaneously induces ROS formation that inhibits RVD (Fig. 5). In this condition no RVD was observed unless the ROS increase was blocked by overexpression of CAT. Finally, Hypo is required to trigger the formation of necrotic blebs which can be inhibited by RVD. Taken together, these data suggest that the Hypo-induced  $\text{Ca}^{2+}$  increase has a dual role in cardiomyocyte: (a) cardioprotective action, activating RVD which inhibits bleb formation; (b) as a cell death inducer by ROS production that inhibits RVD and triggers bleb formation, inducing necrotic cell death (Fig. 5).



**Fig. 5.** Hypotonic stress-induced  $\text{Ca}^{2+}$  increase has a dual role in cardiomyocyte viability. Cells exposed to hypotonic stress increase intracellular  $\text{Ca}^{2+}$  activating ROS formation, inducing RVD which inhibits bleb formation. ROS block RVD which in turn reduces hypotonic stress-induced-necrotic blebs and cell death.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.febslet.2009.10.003](https://doi.org/10.1016/j.febslet.2009.10.003).

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