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Original Paper

Regulatory Volume Increase and Regulatory Volume Decrease Responses in HL-1 Atrial Myocytes

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Key Words

HL-1 atrial myocytes • Cell volume regulation • RVI • RVD • Bumetanide • DIDS

Abstract

Background/Aims: we have investigated whether cultured cardiomyocytes of the cell line HL-1 have the ability to perform regulatory volume responses both in hypotonic and hypertonic conditions. Furthermore, we characterized those regulatory responses and studied the effects of bumetanide and DIDS in volume regulation of HL-1 cells. **Methods:** we used a light scattering system to measure the transient volume changes of HL-1 cells when subjected to osmotic challenge. **Results:** We found that HL-1 cells correct for their volume excess by undergoing regulatory volume decrease (RVD), and also respond to hypertonic stress with a regulatory volume increase (RVI). Rate of RVD was 0.08 ± 0.04 intensity/min, and rate of RVI was 0.09 ± 0.01 intensity/min. Volume recovery was 83.68 ± 5.73 % for RVD and 92.3 ± 2.3 % for RVI. Bumetanide 50 µM inhibited volume recovery, from 92.3 ± 2.3 % (control) to 24.6 ± 8.8 % and reduced the rate of RVI from 0.070 ± 0.020 intensity/min (control) to 0.010 ± 0.005 intensity/min. 50 µM DIDS reduced volume recovery to 42.93 ± 7.7 % and rate of RVI, to 0.03 ± 0.01 intensity/min. **Conclusions:** these results suggest that bumetanide- and DIDS-sensitive mechanisms are involved in the RVI of HL-1 cells, which points to the involvement of the $Na^+/K^+/2Cl^-$ cotransporter and Cl^-/bicarbonate exchanger in RVI, respectively.

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Introduction

The ability to control cell volume is essential for cellular function [1-3]. Mechanisms involved include cell membrane proteins such as ion channels, transporters, ionic pumps and cytoskeletal proteins. Disturbances in cell volume activate several signaling pathways, resulting in adaptive and protective effects in cells [3]. Following an osmotic shock, changes in the volume of cells show two dynamic phases. In the first one, which lasts a few seconds to minutes, water rushes osmotically across the plasma membrane. In the second phase, the cell undergoes a process of volume regulation; induced cell swelling is followed by regulatory volume decrease (RVD), and induced cell shrinking is followed by regulatory volume increase (RVI) [1, 3].

During RVD, osmotically swollen cells release K^+, Cl^-, organic osmolytes, and water [1, 3]. A variety of cell membrane transport mechanisms are involved in RVD such as swelling-activated Cl^- channels, organic osmolyte channels, and swelling-activated K^+ channels [1, 3, 4]. On the other hand, the RVI response occurs by gain of water, Na^+ and Cl^- [1, 3, 4]. In the majority of cells, RVI is mediated by the $Na^+/K^+/2Cl^-$ cotransporter (NKCC), and the Na^+/Cl^- symporter [4-6]. In other cases, an important role is played by the parallel activation of the Na^+/H^+ exchanger (NHE) and $Cl^-/HCO3^-$ anion exchanger [1, 3, 7, 8].

Cardiac cells have been found to regulate their volume by RVD when subject to swelling by hypotonic stress [9]. The RVD response in cardiac myocytes has been studied from the following models: embryonic chicken, neonatal rat, guinea pig, rabbit adult ventricular myocytes and mouse adult atrial myocytes [10-16]. Cultured chick embryo cardiac myocytes [17, 18], ventricular rat myocytes [15], adult rabbit ventricular myocytes [19] and mouse atrial myocytes [16] can exhibit RVD. However, others reported the absence of spontaneous RVD in neonatal rat and guinea pig cardiac myocytes [10, 13, 20, 21]. Evidently there are differences, either between the different cardiac cell models, or in the experimental conditions.

Furthermore, in cardiac myocytes, K^+ channels, Cl^- Channels, and volume regulated anion channels have been described as involved in cell volume regulation [15, 22], and the coordinate action of Cl^- and K^+ channels could be the principal mechanism for RVD [23].

All the transport mechanisms mentioned above in connection with RVI in other cells are also present in cardiac myocytes. However, to date, there are no reports of RVI in cardiac cells [9, 10, 23-25].

In some pathological conditions such as ischemia/reperfusion, hypovolemia, hypernatremia, diabetic shock, or septic shock cardiac cells are exposed to hyposmotic as well as hyperosmotic stress. Alterations in cell volume may seriously affect cardiac function [26]. Cell swelling represents a major threat to the heart by promoting the development of arrhythmias [27]; chronically, it can increase infarct size [28]. In the other hand, hyperosmotic stress reduces cardiac contractibility [29] and can induce apoptotic responses in cardiomyocytes leading to cell death [24]. So, the ability to regulate cell volume is important for the maintenance of cardiac function and homeostasis.

Since we had developed a suitable method to study cell volume changes [30, 31], we thought it would be useful to reinvestigate the situation in cardiac myocytes. We found that HL-1 cardiac myocytes can regulate their volume, both when subject to swelling by hypotonic challenge by regulatory volume decrease (RVD) and when subject to hypertonic stress with a classical regulatory volume increase (RVI). In addition, we found that RVI can be partially inhibited by both bumetanide and DIDS which points to the involvement of the $Na^+/K^+/2Cl^-$ cotransporter and Cl^-/bicarbonate exchanger in the RVI response, respectively.

Materials and Methods

Cell preparation

A murine HL-1 atrial cardiac myocyte cell line was used. Cells were acquired in passage 37 and cultured as described in the literature [32]. Cells were cultured in Claycomb medium supplemented with

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Fig. 1. Schematic diagram illustrating the experimental setup for cell volume measurements. The angular orientation (45°) between incident laser light and coverglass is shown.
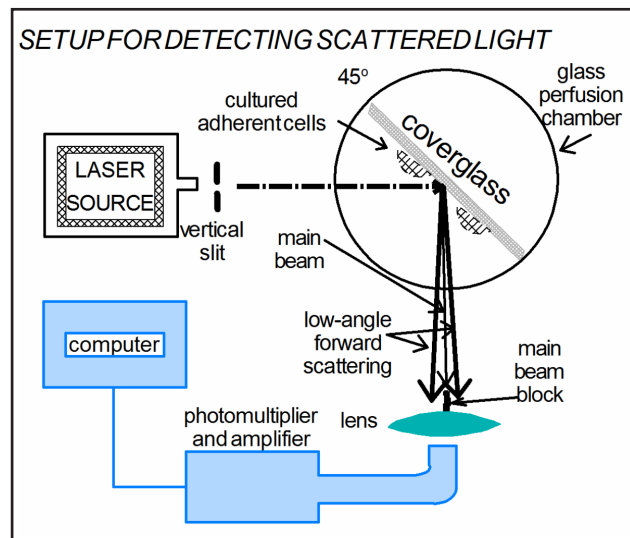
FBS 10 % v/v, streptomycin 100 U/ml, penicillin 100 μg/ml and L-glutamine 2 mM. To harvest cells, we used a solution of trypsine/EDTA 0.05 %/0.02 % (p/v) [32]. For experiments, cells between passages 45-57 were layered on rectangular glass coverslips (11 x 22 mm, Thomas Scientific ®. Cat. # 6663-F10) previously covered by a matrix of gelatine 0.02 % (p/v) - fibronectine 0.5 % (p/v). Coverslips were incubated on culture medium at 37 ºC for 2-3 days to obtain 80 % confluence ($7.5.10^5$ cells/cm^2).

Experimental chamber

Briefly, coverslips with cells were inserted in a plastic plug that has an adjustable slot for this purpose. The plug was placed as the cover for a small cylindrical vessel of glass previously filled with experimental solution. The top of the plastic plug has two openings with tubes of stainless steel (20 gauge) for entry and exit of solutions. Thanks to two rubber O rings, the closure is hermetical, so that movements of liquid are forced. This vessel is maintained at 37 C.

Solutions

The control solution was isotonic Ringer's solution containing (in mM) NaCl 107.9, $NaHCO_3$ 37.0, KCl 4.7, NaH_2PO_4 1.0, $MgSO_4$ (7. H2O) 0.4, $CaCl_2$ (2. H_2O) 1.8, glucose 5.6, HEPES Na^+ 10.0, (pH: 7.4). At the beginning of each experiment, cells were equilibrated for 30 min in control solution. For osmotic challenge, the bathing medium was substituted with 20 % hypotonic (distilled water dilution) or 20 % hypertonic (sucrose added) Ringer's solutions. After each challenge, cells were returned to isotonic solution; recovery took 10-20 min. For inhibition experiments, bumetanide 50 μM and 4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid (DIDS) 50 μM were used. Bumetanide is a specific inhibitor of $Na^+/k^+/2Cl^-$ cotransporter while DIDS is a specific inhibitor of the Cl^-/bicarbonate exchanger. Cells in isotonic Ringer were incubated with the inhibitor for 10 min prior and during osmotic challenge.

Changes of cell volume - light scattering

To estimate changes in cellular volume, we used the intensity of the light scattered by the cultured cells. The method has been described and validated in the literature [30, 31, 33-37]. For illumination, a small laser-diode was used (model L52-265, Edmund Scientific, 4.2 mw, λ = 670 nm), generating a beam of rectangular profile reduced to a width of 1.5 mm by a variable adjustable slit. The coverslip bearing the cells is oriented 45º to the incident laser light beam (Fig. 1). After the main illuminating beam already scattered emerges from the sample, the intensity of light scattered at small angles carries information on cell volume as previously described [30, 36, 37]. This scattered light is captured through a lens endowed with a black plastic (1 mm wide) central band to block the laser main beam, and is measured with a photomultiplier (Hamamatsu R 928) at a fixed gain (-140 V dynode potential). The photomultiplier output (in μAmp) was converted into voltage and amplified through a Chem-Clamp (SN050302, Dagan). This signal was digitized (at 10 KHz) by an analog-digital conversor Digi-data 1440, and it was recorded with Pclamp 10 ® software. Finally, the output of digitized data was normalized as $Y(t) = (I-I_0) / I_0$, where Y(t) is the normalized light

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scatter, I is the experimentally measured light intensity, and I_0 is the basal light intensity (scatter (at rest) in isotonic solution).

Data analysis

Since volume changes result from two time-dependent processes (osmotic and regulatory changes), we fit the data to a double-exponential function. This approach has been used previously [34, 37, 38]. The fitting function is:

$$Y(t) = A_1*(1- \exp(-t/t_1)) + A_2*(1-\exp(-t/t_2))\ (1),$$

where Y(t) is given above, A_1 the (signed) amplitude of the osmotic response, A_2 the (signed) amplitude of the regulatory volume response, t_1 the time constant for the osmotic response, and t_2 the time constant for the volume regulatory response.

The fitting function was used to calculate several parameters of biological interest, namely: the osmotic time, the maximum rate of the osmotic phase, maximum rate of the regulatory phase, the percent of volume recovery, and the maximum value of Y(t).

The osmotic time is defined as the point at which the fitting function reaches its extreme value. It is calculated by taking the derivative of equation (1) and equating it to zero:

$$T_{osm} = \ln(-A1* t2/ (t1* A2))/(1/t1 - 1/t2)\ (2)$$

The maximum rate of the osmotic process is defined as the derivative of the osmotic exponential function evaluated at the initial time, and is calculated as:

$$R_{osm} = A_1 / t_1\ (3)$$

The maximum rate of the regulatory process is defined analogously as the derivative of the regulatory exponential function evaluated at the initial time and is calculated as:

$$R_{reg} = A_2 / t_2\ (4)$$

The percentage of recovery (P_R) is defined as the percentage of the maximum regulatory change respect to the peak value of Y(t) (Y_{peak}). It was calculated as:

$$P_R = 100*(Y_{peak} - Y_{as})/ Yp_{peak}\ (5)$$

where Y_{as} is the asymptotic value of Y(t).

Statistics

All data are given as means ± standard error. Comparisons between treatments were done using a one-way analysis of variance (one-way ANOVA) and contrasts were performed with the Bonferroni test. When two groups were compared a t-test was used. All statistic analyses were performed using Originlab software ®. Statistical significance was set at the conventional 5 % level ($p < 0.05$).

Reagents

Unless noted, all chemicals were purchased from Sigma Aldrich (St Louis, MO, USA). Bumetanide and 4,4′-diisothiocyano-2,2′-stilbene-disulfonic acid (DIDS) were obtained from GIBCO, Life Technologies, USA.

Results

Osmotic responses, and subsequent volume regulatory responses: RVD and RVI

We studied the changes in volume of cultured cardiac myocytes following osmotic challenges with 20 % hypertonic and 20 % hypotonic Ringer solutions.

HL-1 cells responded with swelling after hypotonic challenge, and with shrinkage after hypertonic challenge. Subsequently, as in other cells, we observed that processes of volume regulation developed in both directions; regulatory volume decrease (RVD), and regulatory volume increase (RVI). Figure 2 show the responses of RVI (Fig. 2, upper panel), and RVD (Fig. 2, lower panel) after exposure to solutions 20 % hypertonic or 20 % hypotonic, respectively. Volume regulatory responses took 6–11 min for hypertonic challenge, and 8-15 min for hypotonic challenge; after replenishment in control (isotonic) solution, these regulatory responses were maintained even after repeating the osmotic challenge four times. In addition, volume post-regulatory changes occurred after both the RVI and RVD phases when cells were returned to isotonic solution. Thus, in cells treated previously with

Fig. 2. Cell volume regulation in HL-1 cells. Upper panel. Representative experiment showing a transition from isotonic Ringer to 20 % hypertonic Ringer, and finally, return to isotonic solution in HL-1 cells. Cells initially shrunk and then underwent an RVI response. With the return to isotonic solution, cell swelling occurs and then cells undergo a post-RVI RVD response. The RVI phase of the volume transient lasted some 8-9 min, and the RVD phase, about 7 min. Lower panel. HL-1 cells exposed to 20 % hypotonic medium, initially swell, and then undergo an RVD response. With the return to isotonic solution, the cells shrinked (below their resting volume) and then underwent a post-RVD RVI response. The RVD phase of the volume transient lasted approximately 10 min, and the RVI phase, was incipient and lasted about 3 min.
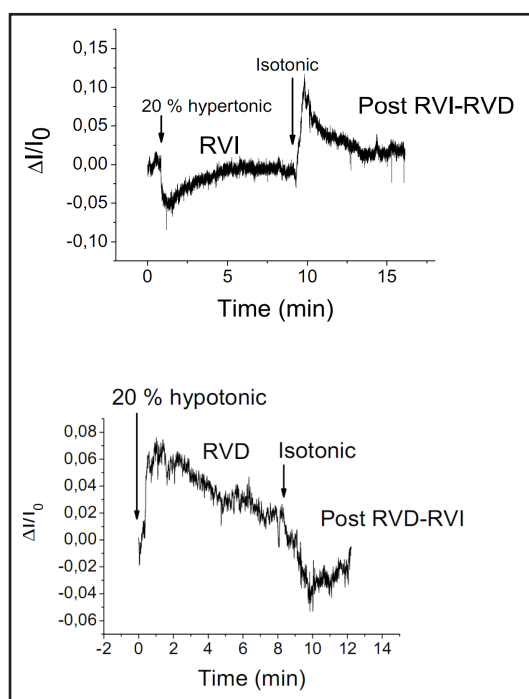
20 % hypertonic solution (Fig. 2, upper panel), there was a post-RVI-RVD after exposure to isotonic solution; and there was a post-RVD-RVI after return to isotonic solution in the cells previously exposed to 20 % hypotonic solution (Fig. 2, lower panel).

Comparison of the RVD and RVI processes

To study the dynamic features of osmotic and regulatory responses, we determined the parameters of double exponential fit to the experimental data (Fig. 3). Thus we obtained the osmotic time T_{osm}, the $\Delta I/I_0$ peak (normalized light scattered light peak after anisotonic challenge, in absolute values at T_{osm}), the rate of recovery of cell volume during RVD and RVI, and the rates for osmotic change and regulatory responses. We found that the osmotic volume changes were definitely faster than the regulatory changes: the rate of osmotic phases (ie: cell swelling/cell shrinkage) was 0.39 ± 0.15 intensity/min for hypotonic challenge (n=6), and 0.46 ± 0.06 intensity/min for hypertonic challenge (n=7) without significant differences between them (Fig. 4). Instead, the rates for the regulatory phases were 0.08 ± 0.04 intensity/min (RVD), (n=6) and 0.09 ± 0.01 intensity/min (RVI), (n=7), although the differences were not significant again (Fig. 5, upper panel). By contradistinction, there was a difference between osmotic rates, and regulatory rates; thus, cells shrink/swell faster than they can regulate their volume by RVI or RVD. The osmotic phase occurs possibly by fast water movements across water channels, while regulatory responses need the slower membrane transporters to work.

The extent of cell volume recovery was near complete in both directions: 83.68 ± 5.73 % and 92.3 ± 2.3 % for 20 % hypotonic (n=6) and 20 % hypertonic solutions (n=7), respectively (Fig. 5, lower panel).

The duration of the osmotic phase T_{osm} was not significantly different in cells exposed to 20 % hypertonic ringer solution (n=7) as compared to cells exposed to 20 % hypotonic Ringer (n=6). T_{osm} was 0.7 ± 0.1 min in 20 % hypertonic treated cells, and 1.3 ± 0.3 min in 20 % hypotonic treated cells (Fig. 4). The degree of cell shrinkage, i.e. the minimum in normalized scattered light $\Delta I/I_0$ (which reflected changes in cell volume) during hyperosmotic exposure was 0.08 ± 0.01 (arbitrary units), (n =7), while the degree of cell swelling (the maximum in $\Delta I/I_0$ during hyposmotic exposure) was 0.07± 0.02 (arbitrary units), (n=6), without significant differences between them (Fig. 4).
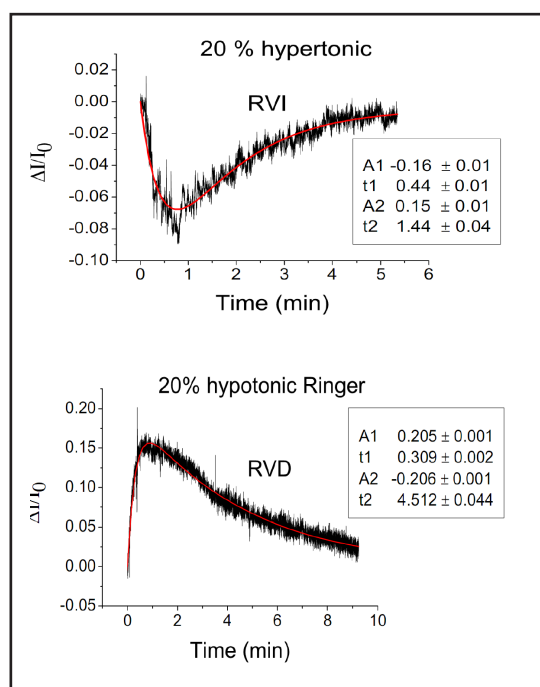
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Fig. 3. Data fit to double exponential function for RVI and RVD in HL-1 cells. Upper panel. Representative experiment showing a RVI response in HL-1 cells after a 20 % hypertonic solution challenge. The regulatory phase of the volume transient lasted approximately 6 min. (RVI), with a cell volume recovery of 98 %. Data was fit to a double exponential function, as described in *Materials and Methods* section. *C*urve fit is shown. The parameters of the double exponential fit are also shown. R^2: 0.97. Lower panel. Representative experiment showing RVD in HL-1 cells after a 20 % hypotonic solution challenge. The regulatory phase of the volume transient lasted approximately 8 min. Volume recovery was 97 % for this experiment. Data was fit to a double exponential function, R^2: 0.97. The parameters of the double exponential fit are also shown. Abbreviations used: A1, amplitude of the osmotic phase; A2, amplitude of the regulatory phase; t1, time constant for the osmotic response; t2, time constant for the regulatory response.
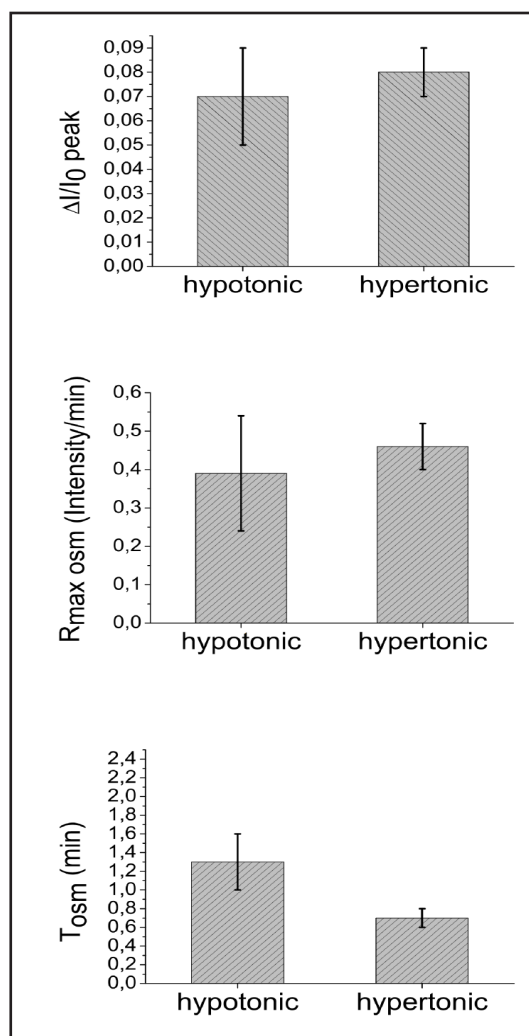

Fig. 4. Characteristics of cell swelling and cell shrinkage of HL-1 cells exposed to 20 % hypotonic and 20 % hypertonic solutions. We show $R_{max\ osm}$ (maximum osmotic rate); T_{0sm} (osmotic time); $\Delta I/I_0$ peak (peak of normalized light scattered light after anisotonic challenge, in absolute values). We didn't find significant differences between hypotonic and hypertonic osmotic challenges at 0.05 significance level.

Bumetanide inhibits the RVI response in HL-1 cardiac myocytes

Since the $Na^+/K^+/2Cl^-$ cotransporter (NKCC) is involved in the RVI responses in many cell types, and is expressed in cardiac cells, we decided to examine the effect of its inhibitor bumetanide on the RVI response. Figure 6 shows a representative experiment. Volume recovery in control experiments (hypertonic alone) was 92.3 ± 2.3 % (n=7), while bumetanide 50 µM significantly inhibited volume recovery, to 24.6 ± 8.8 % (n=5), (Fig.7, upper panel). Bumetanide 50 µM also significantly reduced the rate of RVI from 0.070 ± 0.020 light intensity/min (n=7) under control (hypertonic) conditions, to 0.010 ± 0.005 light intensity/min (n=5) in the presence of the inhibitor, (Fig. 7, lower panel). These results show that a bumetanide-sensitive mechanism is necessary for the RVI response of HL-1 cardiac myocytes.
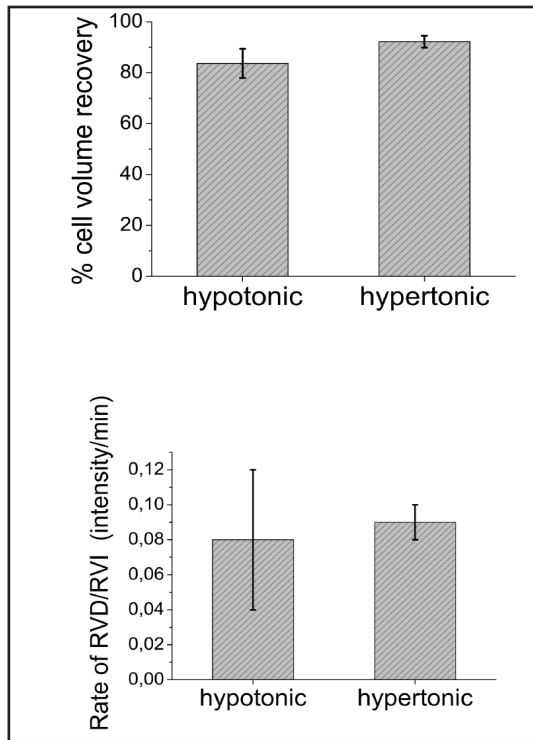
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Fig. 6. Effect of 50 µM bumetanide in the RVI response of HL-1 cells. RVI response was partially inhibited. Note the difference with the complete RVI in Fig 4. Cell volume recovery was 25 % approximately at 10 min. Curve fit to double exponential function is shown. Parameters of double exponential fit are also shown. R^2: 0.96. Abbreviations used: A1, amplitude of the osmotic phase; A2, amplitude of the regulatory phase; t1, time constant for the osmotic response; t2, time constant for the RVD response.

Fig. 5. Volume regulatory responses of HL-1 cells exposed to 20 % hypotonic and 20 % hypertonic solutions. Upper panel. % cell volume recoveries by RVD and RVI are similar for both hypotonic and hypertonic challenge, respectively. Lower panel. Rates of RVD and RVI are similar too.
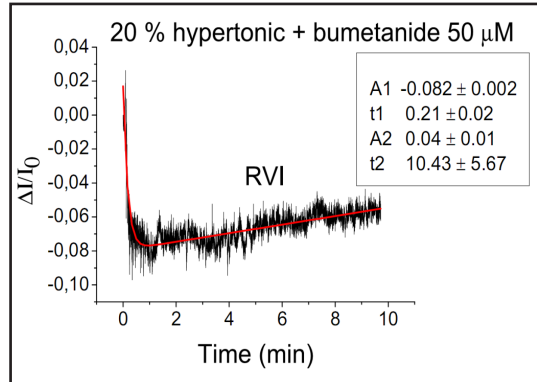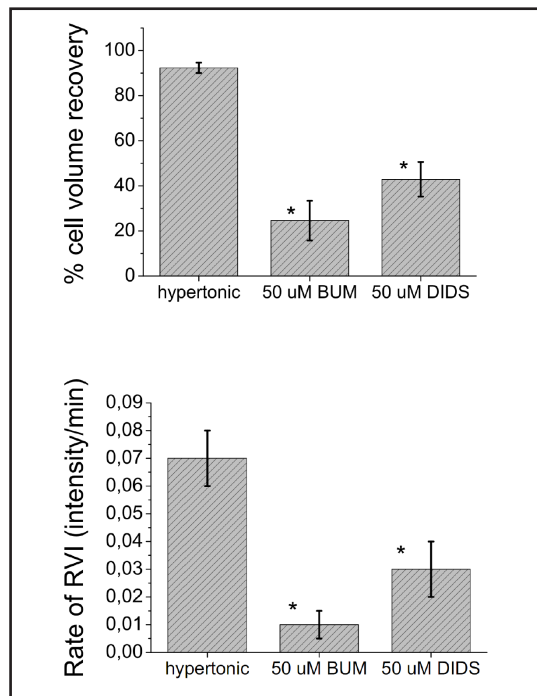

Fig. 7. Inhibition of the RVI response of HL-1 cells exposed to a 20 % hypertonic solution in the presence of 50 µM bumetanide (BUM) or 50 µM DIDS. (*) denotes a significant result (P<0.05).

Bumetanide did not affect the duration of the osmotic phase and the degree of cell shrinkage

The duration of the cell shrinkage phase during hyperosmotic exposure T_{osm} was not significantly different in cells exposed to 20 % hypertonic ringer solution with 50 µM bumetanide (n=5) as compared to cells exposed to 20 % hypertonic Ringer alone (control, n=7). T_{osm} was 0.7 ± 0.1 min in control cells and 0.6 ± 0.2 min in bumetanide treated cells (Fig. 8, upper graph).
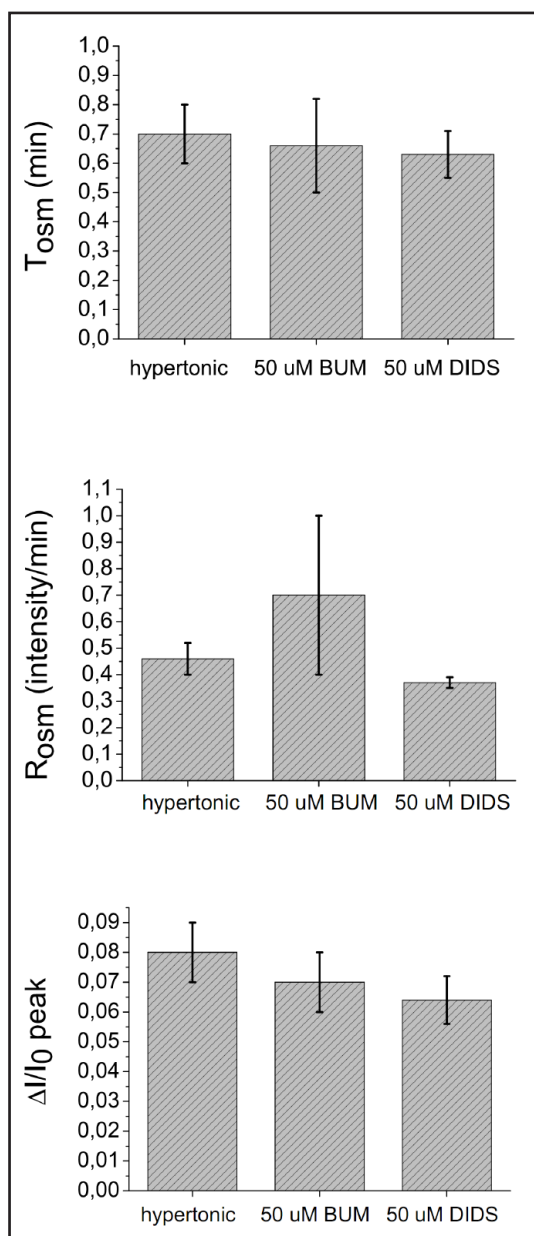
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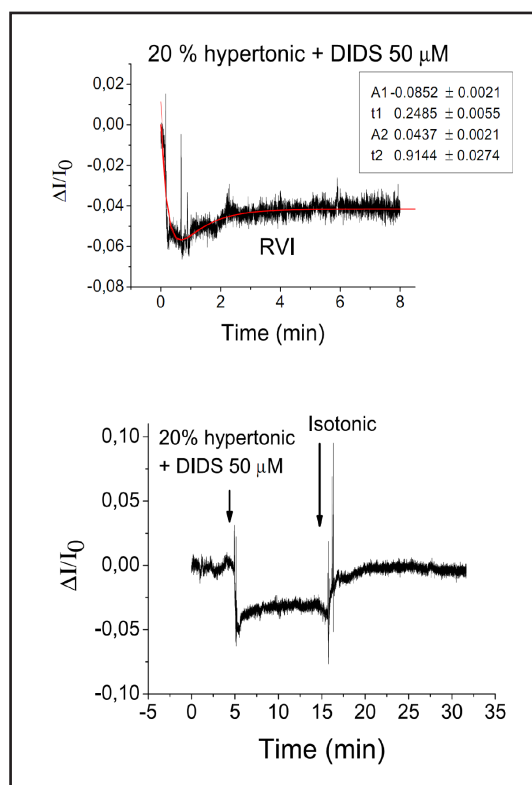Cacace et al.: Cell Volume Regulation in HL-1 Cardiac Myocytes

Fig. 9. Cell volume regulatory responses in DIDS treated HL-1 cells. Upper panel. Effect of 50 µM DIDS in the RVI response of HL-1 cells. Volume recovery at t=8 min was 34 % for this experiment. Curve fit is shown. Parameters of double exponential fit are also shown. R^2: 0.87. Abbreviations used: A1, amplitude of the osmotic phase; A2, amplitude of the regulatory phase; t1, time constant for the osmotic response; t2, time constant for the RVD response. Lower panel. Representative experiment showing a 20 % hypertonic challenge in presence of DIDS 50 µM with additional inhibition of post regulatory RVD. Cells were incubated in isotonic Ringer in presence of 50 µM DIDS for 10 min. and then were exposed to 20 % hypertonic solution supplemented with DIDS 50 µM. RVI was inhibited. No post RVI-RVD was observed after return of cells to isotonic Ringer.

Fig. 8. The osmotic properties of HL-1 cells exposed to a 20 % hypertonic solution was unaffected by 50 µM bumetanide (BUM) or 50 µM DIDS.

Rmax osm was 0.46 ± 0.06 light intensity/min in control experiments and 0.70 ± 0.30 light intensity/min in bumetanide treated cells (Fig. 8, centre graph) without significant differences between them. The degree of cell shrinkage, i.e. the minimum in normalized scattered light $\Delta I/I_0$ (which reflected changes in cell volume) during hyperosmotic exposure, was not significantly different in cells treated with 50 µM bumetanide as compared to control. The minimum of normalized scattered light (maximum cell shrinkage) was 0.08 ± 0.01 (arbitrary units) (n = 7) in control cells (i.e. treated with 20 % hypertonic Ringer alone), and 0.07 ± 0.01 in bumetanide treated cells (Fig. 8, bottom graph).

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DIDS partially inhibits the RVI response in HL-1 cardiac myocytes

To investigate the potential role of the Cl^-/bicarbonate exchanger in the RVI response, we examined the effect of the Cl^-/bicarbonate exchanger inhibitor DIDS. Figure 9 shows representative experiments. DIDS significantly reduced the extent of cell volume recovery by RVI, from 92.3 ± 2.3 % in controls (n=7) to 42.93 ± 7.7 % in DIDS treated cells (n=5), (Fig. 7, upper panel). 50 μM DIDS also significantly reduced the temporal rate of RVI, from an average of 0.07 ± 0.02 light intensity/min under control conditions, to 0.03 ± 0.01 light intensity/min in the presence of 50 μM DIDS, (n=5), (Fig. 7, lower panel). Clearly, a DIDS-sensitive mechanism has a role in the RVI response of HL-1 cardiac myocytes.

DIDS does not have an effect on Tosm and the degree of cell shrinkage in HL-1 cells

T_{osm} and $R_{max\ osm}$ were not significantly different in cells exposed to 20 % hypertonic ringer solution with 50 μM DIDS as compared to control. T_{osm} was 0.7 ± 0.1 min in control cells (n=7) and 0.63 ± 0.08 min in DIDS treated cells (n=5), (Fig. 8, upper graph). $R_{max\ osm}$ was 0.46 ± 0.06 light intensity/min in control experiments, and 0.03 ± 0.01 light intensity/min in DIDS treated cells (Fig. 8, centre graph). The degree of cell shrinkage was not significantly different in cells treated with DIDS 50 μM as compared to control with hypertonic exposure alone. The maximum decrease in the scattered light $\Delta I/I_0$ (arbitrary units) was 0.08 ± 0.01 (n = 7) in control cells, and 0.064 ± 0.008 (n=6) in DIDS treated cells (Fig.8, bottom graph).

Discussion

Osmotic behaviour of HL-1 cells

In this study, we found that HL-1 cardiac myocytes respond to both hypotonic and hypertonic stress by cell swelling and cell shrinkage respectively. There does not seem to be a lot difference between these osmotic responses; the rates of cell swelling and cell shrinkage does not differ significantly in HL-1 cells. In agreement with this, t_{osm} upon hypertonic challenge was in the same order of magnitude than the t_{osm} upon hypotonic challenge. This could be due to the presence of water channels in the plasma membrane of HL-1 cells. It is presumed that the resulting permeability for these channels will be symmetrical for both inflow and outflow. It was previously reported that HL-1 cells express aquaporin 4 (AQP4) in plasmatic membrane [39], which could account for this water movement.

RVD and RVI responses in HL-1 cells

HL-1 cells show RVD response following hypotonic challenge, similar to responses seen in other cardiac atrial cells [16]. Moreover, HL-1 cardiac cells show abundant RVI responses. After repeated osmotic challenges in the same preparation (up to 4 X), cell regulatory responses continued were present and of similar magnitude.

To our knowledge, this is the first time that RVI is reported in isolated cardiac myocytes. There is an observation [40] of rabbit whole heart regaining initial volume after more than 50 min exposure to hypertonic solution, which may point to the presence of RVI in the whole rabbit heart. However, such phenomenon could not be attributed totally to the cardiac myocytes, which represent approximately the 56 % of heart cell composition [41]. Nevertheless, in terms of cell volume myocytes represent about a 75 % of the total volume of the heart [42] so the other cell types could have a lesser contribution to these results.

The current report of RVI in cardiac myocytes applies to a line of long-term cultured cells, which may imply some difference to the more usual fresh non-cultured myocytes or cultured cardiac myocytes. In addition, possibly, volume regulation is more sensitive to some of the experimental conditions such as the solution used, the temperature, or the way cells have been handled. As for RVD, a few authors have reported that it is lost at room temperature [11, 12]. In our HL-1 cell model, both RVD and RVI did not disappear but were merely slowed in time (data not shown) when experiments were carried out at room temperature (25 ºC). In a few cases, after maximum cell swelling/shrinkage, there is a lag period of about 3-6

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min. and then regulatory response starts; in other cases, the cells remain shrunken (data not shown). These observations may explain prior negative RVI observations. In addition, most of the prior research in heart cell volume regulation was done in ventricular myocytes rather than in atrial myocytes. Perhaps there are differences in the ability of different types of cardiac cells to develop a volume regulatory response.

Dynamics of RVD and RVI processes

The volume regulatory responses took approximately 6–11 min for hypertonic challenge, and 8-15 min for hypotonic challenge. The extent of cell volume recovery was similar between RVD and RVI. Furthermore, the rates of both processes were similar too. To be noted, this implies that the dynamics of both regulatory mechanisms are symmetrical even when there are different ionic channels and transporters involved.

Post-regulatory responses in cardiac cells

Following a steady–state RVD, the change of the extracellular environment to an isotonic one promotes cell shrinkage as an osmotic response, and a post RVD-RVI. This response could imply that the prior RVD process had caused a significant decrease in the intracellular ionic concentrations. Similarly, in pathological conditions such as ischemia-reperfusion, cell swelling is observed [43, 44]. It can be argued that such cell swelling might induce an RVD. After these events, reperfusion would act a bathing with isotonic solution, that is, it would induce cell shrinkage followed by a post RVD-RVI. If the reperfusion changes the extracellular ionic concentrations gradually, these processes could occur transiently during ischemia-reperfusion. Typically cell swelling is observed after reperfusion [43, 44], which may be the final consequence of the ionic imbalances that the reperfusion induces.

Regulatory responses in presence of bumetanide 50 µM and DIDS µM: Bumetanide and DIDS inhibit RVI response

Cardiac cells express a large amount of transporters and ion channels, many of which have a function in cell volume down-regulation (RVD) in other cells [9]. Of course it is not known which molecules participate in the RVI of cardiac myocytes. However, it is expected that many of the same transporters and channels that participate in RVI in other cell types would likewise account for the present results.

Sodium-independent chloride-bicarbonate exchangers are present in the plasmatic membranes of many cell types, and function in the regulation of pHi, $[Cl^-]i$, and cell volume. Cardiac myocytes express different isoforms of electrogenic $Cl^-/HCO3^-$ exchangers [45-47], which mediate pH regulation, and possibly contribute to maintenance of $[Cl^-]I$ [47, 48]. These exchangers have been extensively studied in heart muscle in the context of pH regulation. However, the role of $Cl^-/HCO3^-$ exchangers in cardiac cell volume regulation is unknown.

In adition, cardiac myocytes also express the $Na^+/K^+/2Cl^-$ cotransporter [5, 49]. $Na^+/K^+/2Cl^-$ cotransport plays an important role in regulating cell volume in a number of tissues [50-52]. Measurements of cardiac cell volume suggest that $Na^+/K^+/2Cl$ cotransport contributes to both the maintenance of cell volume under isotonic conditions and in response to osmotic stress [10, 53].

In the present study we found evidence for the involvement of the $Na^+/K^+/2Cl^-$ cotransporter and $Cl^-/Bicarbonate$ exchanger in the RVI response of atrial HL-1 cells. According to our results, bumetanide (specific inhibitor of the $Na^+/K^+/2Cl^-$ cotransporter) and DIDS (inhibitor of $Cl^-/Bicarbonate$ exchanger) inhibit the RVI response of HL-1 cardiac cells. Both inhibitors reduce the rate of RVI and as a result the cells present an incomplete regulatory response. The most likely explanation is that in the presence of the inhibitor alternative mechanisms of RVI are still functional. As might have been expected, no effect on RVD was seen in HL-1 cells exposed to 20 % hypotonic solution in presence of 50 µM bumetanide (data not shown); such a result has been previously reported for corneal endothelium cells [37]. Moreover, there is no inhibition of post RVI - RVD in HL-1 cells when

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cells previously exposed to hypertonic solution are returned to isotonic solution (data not shown). On the other hand, post RVI-RVD is inhibited by DIDS (Figure 9, lower panel). This result could be due to inhibition of electroneutral KCl cotransport (KCCs) or Cl^- channels involved in RVD [54; 55]. KCl cotransporters have been described in heart cells [56] and a role was suggested for KCl cotransport in cardiac myocyte RVD [57].

Conclusion

In summary, results of the present study showed that in HL-1 myocytes anisotonic challenge is followed by efficient volume regulatory responses (RVD and RVI), and that RVI can be inhibited by both bumetanide and DIDS, which point to both NKCC and Cl^-/ bicarbonate transporters being involved in the RVI response in these cells. Since the HL-1 cells are phenotypically and functionally similar to adult murine atrial cardiomyocytes [32], these current results could be extended to other atrial cardiomyocytes. Current investigations involve further characterization of these volume regulatory responses, with the aim of determining whether these properties of cardiac cells may be related to pathological conditions.

Abbreviations

RVD, (regulatory volume decrease); RVI, (regulatory volume increase); A_1, (the (signed) amplitude of the osmometric response); A_2, (the (signed) amplitude of the regulatory volume response); t_1, (the time constant for the osmotic response); t_2, (the time constant for the RVD or RVI responses); Y_0, (is the volume at zero time); R_{max}, (average maximum rate for osmotic phase); R_{max}, (average maximum rate for regulatory response); T_{osm}, (osmotic time); DIDS, (4,4'-Diisothiocyano-2,2'-stilbenedisulfonic acid).

Acknowledgments

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