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Volume regulation mechanisms in *Rana castebeiana* cardiac tissue under hyperosmotic stress

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Abstract

Volume changes of cardiac tissue under hyperosmotic stress in *Rana catesbeiana* were characterized by the identification of the osmolytes involved and the possible regulatory processes activated by both abrupt and gradual changes in media osmolality (from 220 to 280 mosmol/kg H_2O). Slices of *R. catesbeiana* cardiac tissue were subjected to hyperosmotic shock, and total tissue Na^+, K^+, Cl^- and ninhydrin-positive substances were measured. Volume changes were also induced in the presence of transport inhibitors to identify osmolyte pathways. The results show a maximum volume loss to $90.86 \pm 0.73\%$ of the original volume (measured as 9% decrease in wet weight) during abrupt hyperosmotic shock. However, during a gradual osmotic challenge the volume was never significantly different from that of the control. During both types of hyperosmotic shock, we observed an increase in Na^+ but no significant change in Cl^- contents. Additionally, we found no change in ninhydrin-positive substances during any osmotic challenge. Pharmacological analyses suggest the involvement of the Na^+/H^+ exchanger, and perhaps the HCO_3^-/Cl^- exchanger. There is indirect evidence for decrease in Na^+/K^+-ATPase activity. The Na^+ fluxes seem to result from Mg^{2+} signaling, as saline rich in Mg^{2+} enhances the regulatory volume increase, followed by a higher intracellular Na^+ content. The volume maintenance mechanisms activated during the gradual osmotic change are similar to that activated by abrupt osmotic shock.
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Keywords: Amphibians; Heart cells; Osmotic stress; Osmolytes; Ion transporters

Introduction

Cell volume is not only important in defining any given cell's external morphology and internal osmolality, but it also affects cellular functions such as membrane transport, metabolism, migration, growth and cellular death (Wehner et al., 2003). A cell in an environment subject to osmotic variations may increase or decrease its volume when under hyposmotic or hyperosmotic challenge, respectively. In order to eliminate the extra volume gained from exposure to a hyposmotic medium, it is necessary for a cell to release solutes (inorganic and/or organic osmolytes) and water. To regain volume lost through exposure to hyperosmotic media, a cell will take up solutes, which is also followed by water. During both types of volume regulatory processes, membrane transport mechanisms are activated (Hoffmanm and Dunham, 1995; Lang et al., 1998).

Although the majority of amphibians are aquatic or semi-terrestrial, they may be found in diverse environments and also inhabit arid regions (Schmidt-Nielsen, 2002).

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An important amphibian characteristic is that they possess damp, permeable skin, which is highly vascularized and participates in breathing and osmoregulation (Pough et al., 1996). Their skin exhibits an evaporative rate similar to free water (Bentley, 1966; Bentley and Yorio, 1979; Hillyard, 1999). Bentley and Yorio (1979) state that in general these animals do not drink water to rehydrate, as this may lead to osmotic stress.

A principal problem for animals that occupy arid environments is evaporative water loss through the body surface. As amphibian urine is very dilute, it may be utilized as a water storage system, but when this is exhausted, and before the animals can activate endocrinal regulation (Rocha and Branco, 1998; DeGrauw and Hillman, 2004), they might well face a dehydration state which promotes an osmotic and ionic concentration of the animal's body fluids (Hillyard, 1999; Schmidt-Nielsen, 2002). This concentration of fluids results in a hyperosmotic stress on different animal tissues. Concerning cell volume, an interesting environmental response has been observed in amphibian freeze tolerance studies which show a correlation between plasma glucose levels and red blood cell volume (Steiner et al., 2000).

Although amphibians are classically known as models of ionic transport in different epithelia (Ussing and Zerahn, 1951), there are no published studies on ionic transport systems associated with cardiomyocyte volume regulation in these animals.

Volume regulation studies in general are performed by submitting cells to an abrupt change in medium osmolality. This probably does not occur in natural environments (Ordaz et al., 2004). Perhaps better models of *in vivo* conditions were first developed by Lohr and Grantham (1986) in the kidney proximal tubule, where cells were exposed to a gradual decrease in medium osmolality. It has been purported that the osmotic shock induced by a gradual change in osmolality may activate regulatory mechanisms different from those activated by a large, single-step change in osmolality (Van Driessche et al., 1997; Souza et al., 2000).

Thus, it is important to evaluate volume changes in amphibian cardiac tissue under hyperosmotic stress, considering a relevant shock that the animals would be expected to experience in their natural habitat. Also, our purpose is to address the osmolytes involved, and the possible regulatory processes activated, by both abrupt and gradual changes in media osmolality.

Material and methods

Experimental model – the animal

Rana catesbeiana, the North American bullfrog, has been introduced into Southern Europe, South America, and Asia, and is now widely distributed. Bullfrogs are very adaptive to different environments, and can tolerate high temperatures. A bullfrog may bury itself in the mud for protection during adverse conditions and to prevent dehydration (Conant, 1975). The species was chosen for this study because it is an animal that faces a broad range of environmental conditions and is relatively large (~0.2 kg, length ranging from 90 to 150 mm), which indicates a relatively large heart.

Cardiac tissue from *R. catesbeiana* (Amphibia, Anura) was chosen for its excitable characteristics, as excitable cells must balance specific ionic requirements for their normal function, with ionic transport necessary for volume regulation. Specimens of *R. catesbeiana* were anesthetized by ice cold water, and their hearts were removed. The ventricle was divided into thin slices of approximately 85 mg (~1 mm thickness) and transferred to isosmotic saline (220 mosmol/kg H_2O), before any of the experimental procedures were performed.

Experimental solutions

The control saline (220 mosmol/kg H_2O, ~pH 7.1) consisted of 111.2 mmol NaCl, 1.4 mmol $CaCl_2$, 1.9 mmol KCl, 2.4 mmol $NaHCO_3$, 3.0 mmol dextrose. Hyperosmotic salines were prepared by concentrating the control solution by 20%, 30% and 50% (265, 280 and 335 mosmol/kg H_2O, respectively).

Cellular volume estimation

Ventricle slices were first incubated for 15 min in a petri dish containing control saline and then carefully blotted on a filter paper, weighed on a balance (1 mg accuracy, Gehaka, BG-200, Brazil) and then immersed in one of the hyperosmotic salines (265, 280 or 335 mosmol/kg H_2O). Following the osmotic shock, the slices were weighed as described above in 15 min intervals. Wet weight changes induced by hyperosmotic conditions were followed for 2 h, and then for 1 h after returning the tissues to control conditions. We compared the above results with the *expected* volume change of the cardiac tissue if it were to display osmometric behavior π_C/π_E, according to van't Hoff's law, where we considered π_C as the osmolality of the isosmotic saline and π_E as the osmolality of the hyperosmotic saline.

In order to evaluate the precision of the wet weighing method used to estimate changes in volume, some ventricle slices were weighed every 15 min for 2:30 h in isosmotic saline (220 mosmol/kg H_2O) as a control. During this period the volume did not change by more than 1.7%. This same procedure has been used in previous studies (Souza and Scemes, 2000; Amado et al., 2006). Any cellular damage resulting from slicing the tissue would not influence our comparisons since it

would be expected to occur equally in the experimental and control groups. If damage were significant in all groups, it would be impossible to achieve a statistically significant difference in our analyses.

The weight change of the muscle slice can be considered to represent major water movements to/from cells of the muscle tissue, and is the effector of proportional changes in volume (Souza and Scemes, 2000; Amado et al., 2006; Freire et al., in press), as also previously inferred in whole animal studies (see Kirschner, 1991; Dunbar and Coates, 2004).

Hyperosmotic shock (280 mosmol/kg H_2O)

After confirming that the heart tissue exhibited volume regulatory mechanisms, we continued the study with only one hyperosmotic solution (280 mosmol/kg H_2O) to study which osmolytes were involved in the regulatory process as well as the regulatory pathways.

Gradual increase in saline osmolality

During the experiments of gradual increase in osmolality a hyperosmotic saline was dripped into the reservoir of isosmotic saline which was flowing onto the preparation, and the slices were weighed every 15 min for 2 h. The slices were incubated in saline with a gradual, continuous change of 0.5 mosmol/kg H_2O/min until the osmotic concentration of the experimental solution reached 280 mosmol/kg H_2O, which marked the end of the experiment.

Inorganic osmolyte analyses

Total Na^+, K^+ and Cl^- contents were determined from heart tissue exposed to isosmotic (220 mosmol/kg H_2O) and hyperosmotic (280 mosmol/kg H_2O) solutions. After these exposures (control, shock and gradual change in osmolality) the slices were transferred to microcentrifuge tubes and then placed in an oven (60 °C) for 24 h. Following the dehydration, 1 ml of nitric acid (HNO_3^-, 0.75 N) was added to each sample to extract the metallic ions. After 24 h the samples were centrifuged and the supernatant was stored at −20 °C until the ionic analysis. One ml of sodium hydroxide (NaOH, 1 N) was added to the pellet for protein solubilization (adapted from Rasmusson et al., 1993); protein concentrations were determined by colorimetric methods (Lowry et al., 1951).

The K^+ and Na^+ content measurements were performed using a flame photometer (Analiser, 900, Brazil), and the Cl^- contents were obtained using a commercial colorimetric kit (Labtest Diagnóstica®, Brazil), employing a spectrophotometer (Shimadzu, UV 1203, Japan). The ion contents were expressed as nmol ion per μg total muscle protein.

Analysis of overall free amino acid content

In addition to analyzing the inorganic osmolytes, we also tested for ninhydrin-positive substances (NPS) in the tissues. For these analyses, we used the colorimetric methods adapted from Clark (1968), as described below.

The slices in the control and experimental groups were weighed to obtain a wet weight, and a homogenate was prepared from each sample in 0.5 ml deionized water. Ethanol (1.25 ml) was then added to each sample, which was centrifuged at 5000g, and the supernatant was preserved for the assay. For the NPS analysis, 0.5 ml samples of the supernatant were reacted with 0.25 ml citrate buffer and 0.6 ml ninhydrin reagent (5 ml of saturated ninhydrin in ethylene glycol and 2 ml of ascorbic acid 1%, and completed to 60 ml with ethylene glycol). The samples were analyzed in a spectrophotometer (Shimadzu, UV 1203, Japan) using glycine as a standard.

Inorganic osmolyte pathway analysis and possible regulators

In order to evaluate inorganic osmolyte fluxes resulting from cell volume regulation we assayed the wet weight changes of heart muscle slices under hyperosmotic saline conditions with blockers of the main ionic transporters activated in regulatory volume increase (RVI). This assay was performed in an attempt to identify the pathways involved in the volume regulatory mechanisms of *R. catesbeiana* cardiac myocytes. The blockers employed were:

Furosemide (at a concentration of $100 \, \mu mol \, l^{-1}$), Sigma Chemical Co. (St Louis, MO, USA): blocker of the Na^+–$2Cl^-$–K^+ cotransporter. 4,4′ Diisothiocyana-tostilbene-2,2′-disulfonic acid (DIDS; at a concentration of $50 \, \mu mol \, l^{-1}$), Sigma Chemical Co. (St Louis, MO, USA): blocker of the Cl^-/HCO_3^- exchanger. Amiloride (at a concentration of $100 \, \mu mol \, l^{-1}$), Sigma Chemical Co. (St Louis, MO, USA): blocker of the Na^+/H^+ exchanger.

Wet weight changes were also analyzed in hyperosmotic Ca^{2+}-free saline and hyperosmotic high Mg^{2+} ($MgSO_4^{2-}$ 0.8 mmol l^{-1}/kg H_2O) saline to evaluate the possible involvement of both of these divalent ions upon the signaling regulatory mechanisms.

Statistical analyses

The mean and the standard error of the mean of the different parameters were analyzed graphically and submitted to Student's *t*-test or analysis of variance (ANOVA) followed by Student–Newman–Keuls multiple range test. We accepted a probability level of $P < 0.05$ as significant.

Results

Cellular volume estimation via wet weight changes

After weighing, heart slices were transferred from 220 mosmol/kg H_2O saline to 265, 280 or 335 mosmol/kg H_2O solution; the maximum weight/volume losses were $86.60 \pm 1.56\%$ (loss of 13.4%; $n = 26$; $P < 0.05$),
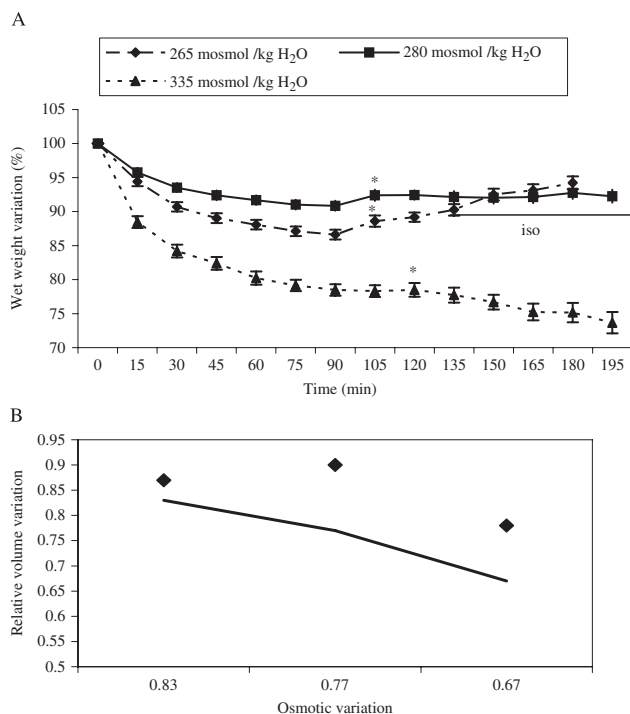

Fig. 1. (A) Time course of *R. catesbeiana* cardiac tissue volume decrease during exposure to 265, 280 and 335 mosmol/kg H_2O hyperosmotic saline. The line indicates the return to isosmotic saline ($n = 26$–31; * $P < 0.05$ compared with time zero). (B) Cardiac tissue volume change expected according to osmometric behavior van't Hoff's law, (π_C/π_E) – solid line; the observed change in the three hyperosmotic salines – diamonds.

$90.86 \pm 0.73\%$ (loss of 9%; $n = 32$; $P < 0.05$) and $78.3 \pm 0.88\%$ (loss of 21.7%; $n = 31$; $P < 0.05$) of the original weight/volumes, respectively (Fig. 1A).

We compared the extent of hyperosmotic shrinkage with that which would be expected based on osmometric behavior (van't Hoff's law). The results are shown in Fig. 1B. The cardiac tissue in Fig. 1 showed weight/volume variations during exposure to hyperosmotic solutions, without recovery, in every situation tested. However, the volume variations shown in Fig. 1B are less pronounced than would be expected for osmometric behavior, which indicates that volume regulatory processes are activated in the cardiac tissue subsequent to exposure to a hyperosmotic medium.

Analysis of inorganic osmolytes

K^+, Cl^- and Na^+ concentrations in cardiac tissue of *R. catesbeiana* during hyperosmotic exposure (280 mosmol/kg H_2O) were determined at 15, 30 and 75 min. Results are presented in Table 1, along with the ion concentration per µg total protein when the tissue was exposed to isosmotic saline, and the percent variation from this value when the tissue was exposed to other experimental conditions.

When compared with the control solution, there was a significant increase in Na^+ contents at all three experimental time points ($P = 0.001$). In contrast, K^+ ion contents decreased at 15 min ($P = 0.000$) and then exhibited a tendency to increase at 75 min ($P = 0.05$). There was no change at 30 min ($P = 0.124$).

Organic osmolytes

Changes in overall free amino acids were analyzed by the determination of NPS. The cardiac tissue was placed under hyperosmotic shock for 75 min and then compared to the control tissue. The NPS content of cardiac tissue in isosmotic saline was 0.988 ± 0.038 µg NPS/mg

Table 1. Total ionic contents in *Rana catesbeiana* cardiac tissue under isosmotic conditions and the relative variations of ionic contents during exposure to hyperosmotic saline (280 mosmol/kg H_2O) for 15, 30 and 75 min; * $P < 0.05$ when compared with controls

Exposure to	K^+			Na^+			Cl^-		
	Mean	SE	*n*	Mean	SE	*n*	Mean	SE	*n*
Isosmotic saline (nmol/µg total muscle protein)	0.24	0.015	29	0.5	0.020	25	0.4	0.016	25
Hyperosmotic saline (280 mosmol/kg H_2O) −15 min (%)	39*	0.007	35*	27*	0.025	33	−0.16	0.21	13
Hyperosmotic saline (280 mosmol/kg H_2O) −30 min (%)	−13.69	0.012	21	18*	0.027	32	6.99	0.23	14
Hyperosmotic saline (280 mosmol/kg H_2O) −75 min (%)	15	0.010	25	24.07*	0.024	27	13.34	0.014	15
Hyperosmotic saline rich in Mg^{2+} 60 min (%)	−57.37*	0.008	8	153.2*	0.138	8	131.22*	0.289	8
Hyperosmotic saline Ca^{2+}-free 60 min (%)	−40.99*	0.007	22	8.68	0.032	27	30.48*	0.17	15

Total ionic contents in cardiac tissue exposed to hyperosmotic saline (280 mosmol/kg H_2O) Ca^{2+}-free and hyperosmotic saline rich in Mg^{2+} (0.8 mmol; $n = 15$–27, $P < 0.05$).

WW (WW = total tissue wet weight) and in hyperosmotic saline, $0.986 \pm 0.049\,\mu g$ NPS/mg WW; results are not significantly different ($n = 6$ for the control group and 7 for the experimental group; $P = 0.496$).

Inorganic osmolyte pathways analyses

We analyzed potential transporters involved in inorganic osmolyte movement during regulatory volume responses in cardiac amphibian tissue, using blockers for the Na^+/H^+ exchanger and the Na^+–$2Cl^-$–K^+, Cl^-/HCO_3^- cotransporters (amiloride, furosemide and DIDS, respectively). After applying hyperosmotic saline ($280\,\mathrm{mosmol}$) with amiloride ($100\,\mu mol\,l^{-1}$) to the tissue, we found a higher maximum weight/volume loss (to $75.08 \pm 0.83\%$ of the original weight/volume; $n = 4$) than in saline without amiloride (to $90.86 \pm 0.73\%$ of the original weight/volume; $n = 32$; $P = 0.000$; Fig. 2). In the presence of furosemide ($100\,\mu mol\,l^{-1}$), the maximum weight/volume loss (to $94.05 \pm 0.79\%$ of the original weight/volume; $n = 10$; $P = 0.05$) was apparently less than in the absence of furosemide (to $90.86 \pm 0.73\%$ of the original weight/volume; $n = 32$; Fig. 2). In order to examine the involvement of the Cl^-/HCO^{3-} exchanger, we applied DIDS ($50\,\mu mol\,l^{-1}$) in hyperosmotic saline. We found that in saline with DIDS, the tissue weight/volume loss (to $94.13 \pm 1.6\%$, $n = 5$) was not significantly different from the control saline (to $90.86 \pm 0.73\%$ of the original weight/volume, $n = 32$; $P = 0.104$).

Analysis of Ca^{2+} and Mg^{2+} in signaling volume regulation

Measuring *R. catesbeiana* cardiac tissue shrinkage in hyperosmotic saline ($280\,\mathrm{mosmol}$) and in the absence of
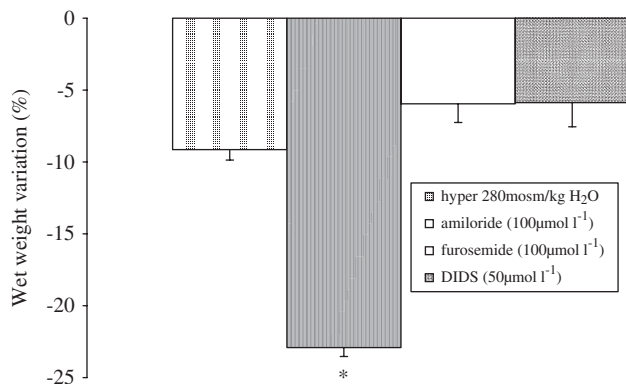
Fig. 2. Maximum volume (expressed as wet weight) variation of cardiac tissue exposed to hyperosmotic saline ($280\,\mathrm{mosmol}/\mathrm{kg}\,H_2O$) and hyperosmotic saline in the presence of $100\,\mu mol$ amiloride, $100\,\mu mol$ furosemide, $50\,\mu mol$ DIDS ($n = 5$–32; * $P < 0.05$ when compared with the hyperosmotic saline without blocker).

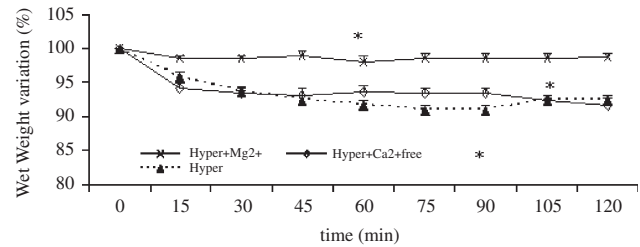**Fig. 3.** Time course of *R. catesbeiana* cardiac wet weight variation when exposed to hyperosmotic saline ($280\,\mathrm{mosmol}/\mathrm{kg}\,H_2O$); hyperosmotic Ca^{2+}-free saline and hyperosmotic saline rich in Mg^{2+} ($n = 8$–32; * $P < 0.05$ when comparing hyperosmotic salines with Ca^{2+}-free and high Mg^{2+}). Hyper = hyperosmotic saline.

extracellular Ca^{2+}, we confirmed that the maximum loss in weight/volume, in the absence of Ca^{2+}, was lower than in its presence (absence: $92.27 \pm 0.92\%$, $n = 32$; presence: $90.86 \pm 0.73\%$, $n = 32$; $P < 0.05$, Fig. 3).

With high Mg^{2+} ($0.8\,\mathrm{mmol}$) hyperosmotic solution, we observed a maximum weight/volume loss (to 98.05 ± 0.73; $n = 8$) that was lower than in the hyperosmotic saline without Mg^{2+} (to $90.86 \pm 0.73\%$; $n = 32$; $P < 0.05$, Fig. 3).

Ionic contents of cardiac tissue exposed to hyperosmotic salines without Ca^{2+} or with high Mg^{2+}

In order to understand the role of Ca^{2+} in the ionic fluxes, cardiac slices were exposed to a Ca^{2+}-free hyperosmotic solution ($280\,\mathrm{mosmol}$) for $60\,\mathrm{min}$. Ion measurements (K^+, Cl^- and Na^+) were compared to the measurements in hyperosmotic saline presented in Table 1. The Na^+ concentration in saline without Ca^{2+} does not differ from isosmotic saline ($P = 0.290$, $n = 27$). Potassium declined in saline without Ca^{2+}, which did not occur in hyperosmotic saline with Ca^{2+} ($P = 0.000$). Chloride also increased in Ca^{2+}-free saline as compared to hyperosmotic solution with Ca^{2+} ($n = 15$; $P < 0.05$).

Ionic concentrations were analyzed after tissue slices were exposed to hyperosmotic saline rich in Mg^{2+} ($0.8\,\mathrm{mmol}$, Table 1). Comparing the saline rich in Mg^{2+} with the hyperosmotic saline without Mg^{2+} one can observe a large increase in Na^+ and Cl^- ($P = 0.00$), while K^+ declined ($P = 0.00$).

Cellular volume analysis during gradual changes in osmolality

In an attempt to mimic osmotic changes that the animal may naturally experience, the cardiac tissue was submitted to a gradual osmolality change ($0.5\,\mathrm{mosmol}/\mathrm{min}$) that began in an isosmotic medium ($220\,\mathrm{mosmol}/\mathrm{kg}\,H_2O$) and ended in a hyperosmotic medium ($280\,\mathrm{mosmol}/\mathrm{kg}\,H_2O$).
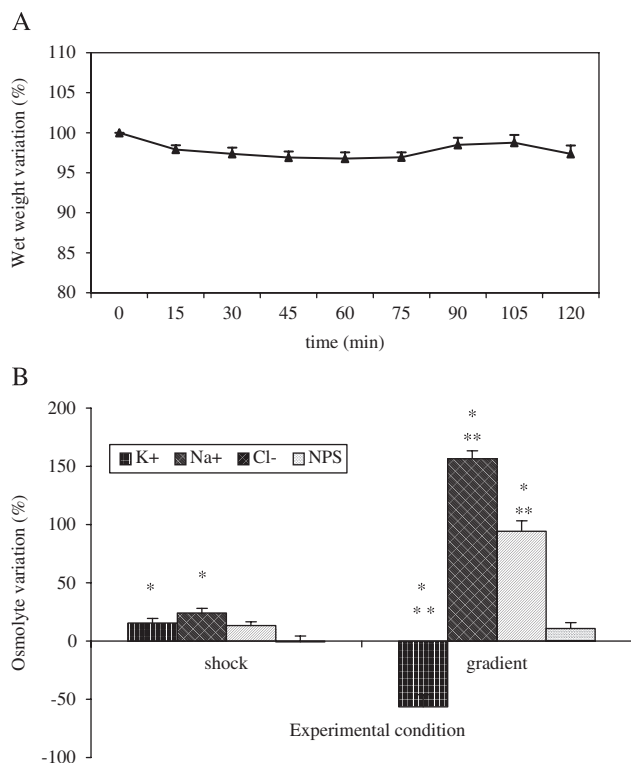
A

B

Fig. 4. (A) Time course of weight/volume variation of *R. catesbeiana* cardiac tissue exposed to a gradual osmolality change ($n = 9$). (B) Ionic content variation in cardiac tissue exposed to hyperosmotic saline (280 mosmol/kg H_2O) for 75 min and at the end of the osmotic gradient ($n = 8$; $P < 0.05$ when different from control *, and when the shock is different from the gradient **).

Ventricle slices of *R. catesbeiana* submitted to the above gradient showed a maximum weight/volume loss to $96.28 \pm 2.2\%$, which was not significantly different from the initial weight/volume under isosmotic conditions ($n = 9$, $P = 0.998$; Fig. 4A).

Ionic contents of cardiac tissue submitted to a gradual change in osmolality

After saline osmolality reached 280 mosmol/kg H_2O (120 min), ionic concentrations were measured. Results are shown in Fig. 4B: after gradient exposure, Na^+ concentrations strongly increased ($n = 8$, $P = 0.00$) when compared with control saline, K^+ decreased ($n = 7$, $P = 0.00$) and Cl^- increased ($n = 8$, $P = 0.00$).

It is important to note that no difference was observed between ionic concentration values (K^+, Na^+ and Cl^-) in this situation (gradual change in osmolality) as compared to the hyperosmotic shock with high magnesium ($P = 0.878$, 0.925, and 0.264, respectively; see Table 1).

Amino acids

Amino acids were also examined (through the analysis of NPS) during a gradual change in osmolality. Results
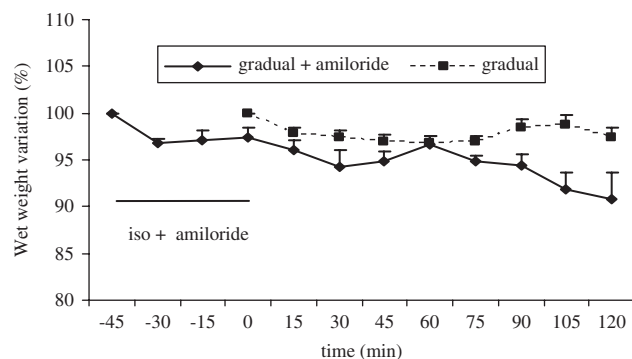

Fig. 5. Time course of volume variation in *R. catesbeiana* cardiac tissue exposed to gradual osmolality increase (0.5 mosmol/min) with and without amiloride ($n = 3–9$).

did not show a significant difference ($P = 0.496$), suggesting no organic involvement in the studied processes during both single-step and gradual osmotic shocks (Fig. 4B).

Na^+/H^+ exchanger participation during an osmotic gradient

Since during hyperosmotic shock only an amiloride-sensitive Na^+ increase was observed, we therefore analyzed the same transporter during a gradual osmolality change. The maximal volume loss in the presence of amiloride was $90.83 \pm 2.83\%$ and thus not different from the weight/volume decrease in the absence of amiloride ($96.28 \pm 2.2\%$, $n = 9$; $P = 0.724$, Fig. 5).

Discussion

Analyses of volume changes indicate that *R. catesbeiana* cardiac tissue activates regulatory mechanisms under hyperosmotic shock, since the results show a lower volume decrease than would be expected if the tissue displayed osmometric behavior (Fig. 1B). When we tested different osmolalities, we observed that 280 and 335 mosmol/kg H_2O induced the highest differences between the expected and the observed volume changes. This behavior suggests a stronger activation of regulatory mechanisms when the cells are under more severe osmotic stress. Returning the slices to isosmotic conditions after exposure to 265 and 280 mosmol/kg H_2O resulted in a notable volume increase, likely in response to changes in intracellular osmolytes.

Osmolyte analyses of *R. catesbeiana* cardiac tissue exposed to hyperosmotic shock suggest the strategy of reducing cellular volume loss by inorganic solute (K^+, Cl^- and Na^+) accumulation (Table 1). During the last few years, it has been increasingly accepted that not only inorganic osmolytes are mobilized in vertebrate cellular osmotic equilibrium recovery, but that organic

osmolytes also play an important role in cell volume homeostasis (Lang et al., 1998; Wehner et al., 2003). However, our organic osmolyte analysis (based on NPS changes) did not reveal any variation. These results may indicate that these cells are regulating volume with electrolytes to prevent the high metabolic debt involved in amino acid synthesis (McCarty and O'Neil, 1992; Souza et al., 2000). The main inorganic osmolyte taken up by the cells during RVI is Na^+; this is a convenient mechanism, as Na^+ is abundant in the extracellular medium. This result led us to investigate which possible transporters may be involved in the inorganic osmolyte pathways.

Based on the analyses of tissue volume during hyperosmotic stress in the presence of amiloride ($100\,\mu mol\,l^{-1}$), furosemide ($100\,\mu mol\,l^{-1}$) or DIDS ($50\,\mu mol\,l^{-1}$) transport blockers of Na^+/H^+, $Na^+–2Cl^-–K^+$ and HCO_3^-/Cl^-, respectively, we can discuss the participation of each osmolyte pathway in the regulatory process of RVI. When amiloride ($100\,\mu mol\,l^{-1}$) was applied during a hyperosmotic shock, we observed a probable involvement of the Na^+/H^+ exchanger (NHE) in *R. catesbeiana* cardiac tissue volume regulation, since the maximum volume loss was greater than in the hyperosmotic saline of 280 mosmol/kg H_2O (Fig. 2). Red blood cell shrinkage in *Amphiuma* also implicates the Na^+/H^+ exchanger as important in the regulation of cell volume (Cala, 1980; Cala and Maldonado, 1994). Gusev and Ivanova (2003) observed Na^+ influx inhibition in *Rana ridibunda* red blood cells during hyperosmotic stress, which also corroborates the interpretation of our results.

Our results did not show evidence of the Cl^-/HCO_3^- (anion exchanger, AE) transporter's participation, as we could not measure a parallel increase of Cl^- with Na^+ (at 30 min) which may support the hypothesis that the Cl^-/HCO_3 exchanger is not involved, nor perhaps any other Cl^- pathway. The $Na^+–2Cl^-–K^+$ cotransporter (NKCC) is also probably not involved in the hyperosmotic RVI of *R. catesbeiana* cardiac tissue, although NKCC in this cell type did not seem to be sensitive to furosemide. Based only on ion content results, it is likely that Na^+/K^+-ATPase is active during all experimental times tested. At 75 min, the ATPase activity could possibly be re-established to activate the original Na^+ gradient restoration, in parallel with a lower Na^+/H^+ participation.

Calcium involvement in volume regulation has been recorded in many cellular systems, and calcium signaling is widely reported after cell swelling triggers the regulatory volume response (McCarty and O'Neil, 1992; Strange, 1994; Hoffmanm and Dunham, 1995; Wehner et al., 2003). The same is not normally observed during RVI (Marchenko and Sage, 2000; Erickson et al., 2001). Cell swelling may lead to an increase in intracellular Ca^{2+} by influx activation, or by storage release, or both (Lang et al., 1998; Souza et al., 2000). Our results obtained with *R. catesbeiana* are not an exception, and there was no evidence of an extracellular Ca^{2+} function in signaling the RVI (Fig. 3).

Our results show that *R. catesbeiana* cardiac tissue maintains its cellular volume in hyperosmotic solution rich in Mg^{2+}, which seems to be a consequence of a higher accumulation of Na^+ and Cl^- (Fig. 3 and Table 1). Magnesium can activate or inhibit many transport systems, but inhibition due to high ion concentration may be by substrate competition (Birch, 1993). It has been shown that physiological magnesium changes have a biphasic effect on sodium ATPase activities and K^+-Cl^- cotransporters (Flatman, 1993). Considering that *R. catesbeiana* cardiac tissue maintained its volume in saline rich in Mg^{2+}, and that it showed a K^+ loss and an accumulation of Na^+ and Cl^-, we suggest that the Na^+/K^+ ATPase transporter might be inhibited (by Mg^{2+} competition for the beta subunit) and the Na^+/H^+ exchanger may be activated.

Gradual osmolality change

The effect of a gradual change in osmolality on cardiac tissue volume was analyzed, and tissue volume remained constant (Fig. 4A), probably due to inorganic solute (Na^+ and Cl^-) accumulation, which is greater in isovolumetric regulation (IVR) than in RVI. Different studies have shown that during a gradual change in osmolality cells may activate regulatory mechanisms more efficiently, so that volume changes are minimal or even absent (Lohr and Grantham, 1986; Van Driessche et al., 1997; Souza et al., 2000; Ordaz et al., 2004).

Mechanisms involved in cellular volume regulation during hyperosmotic shock and gradual osmolality increase seem to be dynamically different, but apparently use the same pathways for solute accumulation. The slow change in osmolality may signify a lower stress for the cells so that they are able to activate their regulatory mechanisms to avoid volume variations during the osmotic shock; the greater stress may demand a higher level of signaling (by Mg^{2+}) to perform the necessary solute uptake to minimize changes in volume.

Considering the animal in a natural environment with water deprivation and subsequently a gradual solute accumulation in its plasma, it seems very likely that *R. catesbeiana* cardiomyocytes will exhibit cell regulatory mechanisms to maintain a steady volume.

Acknowledgments

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