Biphasic effects of hyposmotic challenge on excitation-contraction coupling in rat ventricular myocytes

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cardiac myocyte; hyposmotic swelling; action potential; sarcoplasmic reticulum; L-type calcium current

IN THE HEART, cell swelling occurs, for example, during an acute myocardial ischemia-reperfusion episode. During ischemia, metabolites, such as lactate, accumulate within the cell and can cause swelling. Swelling may be further exacerbated on reperfusion when the hyperosmotic extracellular milieu is exchanged for blood with normal osmolality, creating a significant osmotic gradient between the extracellular and intracellular space.

Research has shown that cell swelling has many electrophysiological consequences (for reviews, see Refs. 6 and 32). Anion conductance and nonspecific cation channels activated by osmotic cell swelling have been described using both conventional whole cell (17, 31, 33) and perforated patch-clamp techniques (7, 31). These channels are probably inactive in the absence of swelling. For example, Cl− current activated by swelling (I_{Cl,Swell}) has been shown to participate in the diastolic membrane depolarization associated with cell swelling in both atrial and ventricular cells (10, 35). In guinea pig ventricular myocytes, a biphasic effect of swelling on the action potential duration (APD) has been observed, with an initial increase in APD followed by a subsequent decrease (35). I_{Cl,Swell} has also been shown to participate in the delayed abbreviation of the APD (35).

Swelling has been shown to affect time-dependent currents, which normally contribute to action potential configuration. With the use of a whole cell patch-clamp technique, the slow delayed rectifier K+ current has been shown to be markedly increased by cell swelling in both guinea pig and canine myocytes, whereas the rapid delayed rectifier K+ current (I_{K_r}) is reduced during swelling in guinea pig cells (24). The L-type Ca2+ current (I_{Ca,L}) has been shown to be either insensitive, increased, or decreased by cell swelling (see Ref. 6 for a review). For example, using the whole cell patch-clamp technique, I_{Ca,L} has been shown to be insensitive to cell swelling in guinea pig (13, 26) and dog (40) ventricular myocytes and increased in rabbit sinoatrial cells after 2 min of swelling (23). Recently, Li and Baumgarten (22) reported a biphasic effect of cell swelling on I_{Ca,L} in rabbit ventricular myocytes by using the perforated patch-clamp technique. They observed a transient increase in I_{Ca,L} after a 2-min exposure to a hyposmotic solution, which was followed by a subsequent decrease in I_{Ca,L} after 10 min exposure.

Swelling differs from other forms of membrane deformation, such as axial stretch, because it involves the dilution of intracellular contents. There is limited information on changes in the intracellular milieu during swelling. Dilution of intracellular potassium under-

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neath the sarcolemma has been associated with cell swelling (1, 10). A decrease in intracellular Na⁺ activity has been observed in both sheep and rabbit ventricular muscle (19, 37). Stimulation of the Na⁺-K⁺-ATPase after swelling has been noted in rabbit ventricular myocytes, an effect that was ascribed to an increase in the affinity of the pump for Na⁺ (37). The only study (19) that has examined the effect of hyposmotic challenge on intracellular Ca²⁺ concentration ([Ca²⁺]i) observed a decrease in the average level of [Ca²⁺], in sheep Purkinje fibers, which was thought to arise secondary to the reduced intracellular Na⁺ activity. A corresponding reduction in the force of contraction was noted in sheep ventricular muscle (19).

Despite the body of information that has accumulated regarding the electrophysiological consequences of cell swelling, there is only limited information concerning the effect of swelling on excitation-contraction (E-C) coupling in the cardiac cell. The relationship among hyposmotic-induced changes in electrical properties, intracellular ion concentrations, sarcoplasmic reticulum (SR) function, and mechanical function have not been studied previously in a single cell system. In the present study, therefore, we investigated the effect of hyposmotic challenge on the action potential, ICa,L (the major trigger for Ca²⁺ release), SR function, [Ca²⁺], transients, and contraction of single rat ventricular myocytes. Evidence suggests that several of the effects of swelling are time dependent; therefore, we studied changes in these parameters after both a short and prolonged exposure to hyposmotic solution.

**METHODS**

**Isolation of rat cardiac ventricular myocytes.** Single rat ventricular myocytes were isolated according to the method of Frampton et al. (11). Briefly, male Wistar rats (200–250 g) were killed in accordance with the home office Guidance on the Operation of the Animals (Scientific Procedures) Act of 1986. Hearts were removed quickly and mounted on a Langendorff apparatus. Hearts were perfused retrogradely with a HEPES-based isolation solution of the following composition (in mM): 130 NaCl, 5.4 KCl, 0.4 NaH₂PO₄, 1.4 MgCl₂, 6H₂O, 0.75 CaCl₂, 10 HEPES, 10 glucose, 20 taurine, and 10 creatine (pH 7.3). When the coronary circulation had cleared of blood, perfusion was continued with Ca²⁺ and 10 creatine (pH 7.3). When the coronary circulation had

**Measurement of cell length and [Ca²⁺].** To measure cell length and [Ca²⁺], simultaneously, myocytes were illuminated with red light (λ = 610 nm) to generate an image of the cell detected by a camera mounted on the microscope and displayed on a monitor. The length of this image was monitored using an edge detection system (Crescent Electronics), and the change in cell length during stimulation was used as our index of contractility. The image of the cell also allowed us to estimate changes in cell volume during swelling, according to the equation V = (Iw/wd/Ld) (where V is volume, w is width, d is depth, and L is length, assuming the cell is an elliptical cylinder with a ratio of width to depth of 3:1) (4).

Cells were loaded with the Ca²⁺-sensitive fluorescent indicator fura 2-acetoxyethyl ester (AM) (Molecular Probes) by incubation in 0.75 mM Ca²⁺ isolation solution containing 3–5 μM fura 2-AM for 10 min at room temperature. The ratio of fluorescence emitted at 510 nm in response to alternate excitation with light of 340 and 380 nm (340/380 ratio) was used as an index of the intracellular Ca²⁺ activity. Effect of cell swelling on fura 2 fluorescence. Several studies (34, 39) have shown that a decrease in ionic strength in vitro can increase the affinity of fura 2 for Ca²⁺. The magnitude of decrease in ionic strength is pivotal in this respect. Wong et al. (39) showed that for reductions in ionic strength up to 15%, there was no change in the dissociation constant (Kₐ) of fura 2. For reductions in intracellular ionic strength above this value, we must consider the possibility that the Kₐ of fura 2 will be decreased and, therefore, give an increase in the magnitude of the fura signal for a given level of [Ca²⁺].

For this reason, when the degree of swelling could result in a dilution of intracellular contents by >15%, we interpreted any change in fura 2 fluorescence other than a decrease with caution. A decrease in the fura 2 signal can be assumed to represent a true qualitative change in [Ca²⁺]. The ratio of two fura-based signals measured at the same ionic strength (for example, in determination of fractional SR Ca²⁺ release) should not be influenced by an effect of ionic strength on fura 2 fluorescence.

**Measurement of action potentials and ICa,L.** Action potentials and ICa,L were measured in the whole cell configuration of the patch-clamp technique using an Axopatch 2B amplifier (Axon Instruments) controlled by a CED 1401 interface and software (Cambridge Electronic Design). Glass pipettes were pulled from nonheparinized hematocrit tubes to a tip resistance of 2–5 MΩ. Cells were superfused by solution as indicated. For action potential recordings, the pipette solution had the following composition (in mM): 110 potassium aspartate, 10 KCl, 10 NaCl, 8 MgCl₂, 8 Kₐ-ATP, 10 HEPES, and 0.05 EGTA (pH adjusted to 7.1 with KOH). For ICa,L recordings, the pipette solution had the following composition (in mM): 130 CsCl, 10 NaCl, 8 MgCl₂, 8 Kₐ-ATP, 10 HEPES, 5 glucose, and 5 1,2-bis(2-aminophenoxy)ethane-N,N',N''-tetraacetic acid (pH adjusted to 7.2 with CsOH).

Action potentials were elicited by 2-ms current steps just above threshold. To measure ICa,L, cells were voltage clamped at a holding potential of −80 mV. A 500-μs prepulse to −40 mV was used to inactivate Na⁺ current, and ICa,L was then elicited by a 300-ms step pulse from −40 to 0 mV. The amplitude of ICa,L was measured as the difference between peak inward current and the current remaining at the end of the 300-ms voltage-clamp pulse. Stimulation frequency was 0.33 Hz.
Confocal microscopy and measurement of t-tubular distribution. To visualize the cell membrane and t-tubular system after hyposmotic challenge, cells were exposed to isosmotic or hyposmotic solution for 10 min and then loaded with the lipophilic voltage-sensitive fluorescent indicator di-8-aminonaphthylethenylpyridinium (di-8-ANNEPS, 5 μM; Molecular Probes) for 2 min (16). Cells were resuspended in either isosmotic or hyposmotic solution and imaged within 20 min using confocal laser scanning microscopy (Leica True Confocal Scanner SP) with the use of 488-nm excitation light with detection at 514 nm. The spacing of the t-tubules in each cell was calculated by dividing the number of striations (the t-tubular invaginations) by the length of the cell.

Quantitative immunoblotting and measurement of the phosphorylation of phospholamban. To measure the site-specific phosphorylation of the SR protein phospholamban, aliquots of cells were suspended in either isosmotic or hyposmotic solution and placed in wells of a Perspex container. Cells were stimulated at 0.5 Hz and gently agitated to prevent settling. After 5 min had passed, cell suspensions were dispersed in sample buffer to give a final concentration of 26.2 mM Tris-HCl (pH 6.8), 10% glycerol, 5% 2-mercaptopropanol, 2% SDS, and 0.1% bromophenol blue. Myocardial proteins were separated by SDS-PAGE using 15% acrylamide gels. Phosphorylation of phospholamban at residue 16 (Ser165) and 17 (Thr17) was quantified using the method described by Calaghan et al. (5). In brief, a standard calibration curve was constructed using the peptide PL919Y (which consists of residues 9–19 of phospholamban with a COOH-terminal tyrosine, phosphorylated at either Ser165 or Thr17), which was run in parallel with cell samples. After they were separated, proteins were transferred to polyvinylidene fluoride membranes by semidy blotting, and membranes were then probed overnight at 4°C with primary antibodies to the Ser165 (1:10,000) and Thr17 (1:5,000)-phosphorylated forms of phospholamban (9). Immunoreactivity was visualized using a peroxidase-based chemiluminescence substrate kit (Amer sham). Bands on the autoradiographs were quantified by whole-band densitometry.

Solutions. Cells were exposed first to an isosmotic solution (osmolarity 290 ± 5 mosM), which had the same composition as the standard physiological solution (osmolarity 280 ± 5 mosM) except that the NaCl concentration was reduced to 57 mM NaCl and 113 mM sucrose was added. To induce an osmotic shock, cells were exposed to an hyposmotic solution having the same composition as the isosmotic solution but without sucrose (osmolarity 180 ± 5 mosM). For all solutions, pH was adjusted to 7.4, and osmolarity was measured using a Roebelng osmmeter. A rapid switching device was used to locally superfuse the cell under investigation and to allow exchange of solutions rapidly (within 1 s) (20). When it was used, caffeine (10 mM) was dissolved directly in the experimental solutions. Stock solutions (1 mM) of di-8-ANNEPS were made up in dimethylsulfoxide containing 20% (wt/wt) pluronic acid and stored at −20°C.

Statistical analysis. Data were expressed as means ± SD of n observations. Statistical analysis was performed using the paired Student’s t-test or ANOVA as appropriate. P values <0.05 were considered significant.

RESULTS

Effects of substituting NaCl with sucrose on E-C coupling. We first determined the effects of NaCl substitution with sucrose on action potential duration at 90% repolarization (APD90), I_{Ca,L}, [Ca^{2+}]_i, and contraction. Mean data are given in Table 1. In isosmotic solution, APD90, I_{Ca,L}, diastolic [Ca^{2+}]_i, [Ca^{2+}]_i, transient amplitude, and contraction were significantly (P < 0.05) increased compared with those recorded in standard physiological solution. The inotropic effects of solution changes were also observed in whole cell patch-clamped cells.

Effects of hyposmotic challenge on cell volume. We recorded the effect of exposure to hyposmotic solution on cell length and width compared with that recorded in isosmotic solution. Cell volume was calculated according to the method of Boyett et al. (4) (see METHODS). In isosmotic solution, in a representative population of eight cells, the mean dimensions were length 103 ± 5 μm, width 25.4 ± 2.0 μm, and volume 20.5 ± 3.6 pl. We saw no significant increase (P > 0.05) in length (0.17 ± 0.17%, n = 5), width (5.1 ± 3.4%), or volume (11.0 ± 7.3%) at 1 min after hyposmotic challenge. The major change in cell dimension after 10-min exposure to hyposmotic solution was an increase in cell width of 15 ± 2% (n = 8, P < 0.001), whereas cell length only increased by 3.3 ± 1.1% (P < 0.05). The increase in cell volume was 38 ± 6% (P < 0.001). Although we did not measure the volume change for every cell, swelling was verified visually in all cases after long exposure to hyposmotic solution. Given the magnitude of increase in cell volume after long exposure to hyposmotic solution, we cannot exclude the possibility that dilution of intracellular contents changed the relationship between fura fluorescence and [Ca^{2+}]_i (see Ref. 39 and METHODS).
solution. In contrast, under these conditions, the amplitude of $I_{Ca,L}$ was significantly reduced ($P < 0.01$). After short hyposmotic challenge, diastolic fura signals were unchanged. However, at this time point, the amplitude of the fura 2 transient was significantly higher ($P < 0.001$). At 1 min after exposure to hyposmotic solution, we saw no change in resting membrane potential (isosmotic solution $-77.2 \pm 1.0$ mV and hyposmotic solution $-77.4 \pm 1.0$ mV, $n = 12$).

Representative traces showing the effects of long hyposmotic challenge are given in Fig. 2, and mean data are given in Table 2. In contrast to the situation seen after short exposure to the hyposmotic solution, after 10 min of hyposmotic challenge both APD$_{90}$ and contraction amplitude were significantly reduced ($P < 0.05$). A further depression in the amplitude of $I_{Ca,L}$ was seen at this time point ($P < 0.05$ compared with both isosmotic and short hyposmotic values). There was no significant effect ($P > 0.05$) of long hyposmotic challenge on diastolic fura 2 signals, whereas the fura transient amplitude was significantly lower ($P < 0.001$) than those recorded in isosmotic conditions. We saw a significant ($P < 0.05$) depolarization of the resting membrane potential (isosmotic solution $-77.2 \pm 1.0$ and hyposmotic solution $-75.8 \pm 1.1$ mV, $n = 10$) after long exposure to hyposmotic solution.

After 10 min of hyposmotic challenge, some cells were superfused with isosmotic solution. Cell size returned to control levels after return to isosmotic solution. The effects of hyposmotic solution were irreversible for all other measured parameters.

**DIDS sensitivity of APD$_{90}$ reduction.** Because it has been reported that, in guinea-pig ventricular cells, the reduction of APD after long exposure to hyposmotic solution was increased.

### Table 2. Effects of short and long hyposmotic challenge on APD$_{90}$, $I_{Ca,L}$ amplitude, diastolic [Ca$^{2+}$]$_i$, [Ca$^{2+}$]$_i$ transient amplitude, and contraction in rat ventricular myocytes

<table>
<thead>
<tr>
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<th>Short Hyposmotic Challenge</th>
<th>Isosmotic</th>
<th>Hyposmotic</th>
<th>Long Hyposmotic Challenge</th>
<th>Isosmotic</th>
<th>Hyposmotic</th>
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<tr>
<td>APD$_{90}$, ms</td>
<td>44 ± 13(12)</td>
<td>51 ± 16(12)#</td>
<td>42 ± 11(9)</td>
<td>37 ± 12(9)</td>
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<tr>
<td>$I_{Ca,L}$, pA</td>
<td>888 ± 64(7)</td>
<td>493 ± 44(7)†</td>
<td>888 ± 64(7)</td>
<td>173 ± 43(7)‡</td>
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<tr>
<td>Diastolic [Ca$^{2+}$]$_i$, ratio units</td>
<td>0.162 ± 0.018(17)</td>
<td>0.163 ± 0.018(17)</td>
<td>0.162 ± 0.018(17)</td>
<td>0.158 ± 0.018(17)</td>
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<tr>
<td>[Ca$^{2+}$]$_i$, transient, ratio units</td>
<td>0.119 ± 0.012(33)</td>
<td>0.131 ± 0.014(33)‡</td>
<td>0.121 ± 0.014(30)</td>
<td>0.080 ± 0.008(30)‡</td>
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<tr>
<td>Contraction, % cell length</td>
<td>7.05 ± 0.99(16)</td>
<td>8.97 ± 1.17(16)*</td>
<td>6.85 ± 1.01(13)</td>
<td>3.97 ± 0.90(13)*</td>
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Values represent means ± SE, with the number of observations shown in parentheses. Short hyposmotic data were recorded at 50–70 s after perfusion with hyposmotic solution, and long hyposmotic data were recorded between 7 and 10 min of perfusion with hyposmotic solution. *$P < 0.05$, †$P < 0.01$, and ‡$P < 0.001$ compared with those recorded in respective isosmotic conditions (paired Student’s $t$-test).
solution is due to the activation of $I_{Cl,Swell}$, we tested the effects of the inhibitor of $I_{Cl,Swell}$, DIDS (50 µM), on the action potential in isosmotic and hyposmotic conditions. In isosmotic solution, exposure to 50 µM DIDS for 30 s had no effect on the action potential, as indicated in Fig. 3A. In contrast, when cells were swollen after hyposmotic challenge, 50 µM DIDS partially reversed the APD reduction at all time points studied (after 3, 6, and 9 min of superfusion) in three of three cells (Fig. 3B).

Effects of cell swelling on t-tubules. It has been shown recently (16) that an osmotic shock, produced by washing out formamide from cells previously superfused with this agent for 15 min, causes de-t-tubulation of rat ventricular cardiac myocytes, a reduction in $I_{Ca,L}$, and a fall in SR Ca\(^{2+}\) release. In the present study, it is possible that the fall in amplitude of $I_{Ca,L}$ upon exposure to hyposmotic solution was the result of swelling-induced de-t-tubulation. Figure 4 shows confocal images of cells exposed to either isosmotic or hyposmotic solution for 10 min, labeled with the voltage-sensitive dye di-8-ANPEPS. In cells exposed to hyposmotic solution, large sarcolemmal “blebs” were evident, but the regular t-tubular spacing pattern was clearly visible, and there was no evidence of “missing” striations. The spacing between the t-tubules was significantly greater by 4.6% in cells in hyposmotic solution, large sarcolemmal “blebs” were evident, but the regular t-tubular spacing pattern was clearly visible, and there was no evidence of “missing” striations. The spacing between the t-tubules was significantly greater by 4.6% in cells in hyposmotic solution (1.96 ± 0.03 µm, n = 24) compared with those in isosmotic solution (1.87 ± 0.01 µm, n = 27). This increase in t-tubular spacing is consistent with the observed swelling-induced increase in cell length (3.3 ± 1.1%). Therefore, the fall in $I_{Ca,L}$ after swelling could not be ascribed to a fall in available Ca\(^{2+}\) channels as a result of de-t-tubulation.

Effects of hyposmotic challenge on SR function. To test whether the effects of hyposmotic challenge could be ascribed to an action on SR function, we examined the effect of caffeine on SR Ca\(^{2+}\) release in hyposmotic conditions. A representative trace illustrating the protocol is shown in Fig. 5A. Experiments were performed initially in isosmotic conditions. Steady-state fura 2 transients were recorded in response to electrical stimulation, stimulation was then switched off, and, after a delay of 10 s, 10 mM caffeine was rapidly applied to the cell. This protocol was repeated after either 1 or 10 min of perfusion with hyposmotic solution. In agreement with data given earlier, the amplitude of electrically induced transients was significantly greater ($P < 0.05$) after short hyposmotic challenge (0.124 ± 0.008 vs. 0.112 ± 0.008 ratio units, n = 16) yet significantly depressed ($P < 0.05$) after long hyposmotic challenge (0.075 ± 0.006 vs. 0.113 ± 0.013 ratio units, n = 13). After short hyposmotic challenge, caffeine-induced transients (indicative of SR Ca\(^{2+}\) load) were the same as those in isosmotic conditions (0.146 ± 0.010 vs. 0.146 ± 0.011 ratio units, n = 16). After long hyposmotic challenge, caffeine-induced fura transients were smaller than those in isosmotic conditions, although the difference was not significant (0.129 ± 0.010 vs. 0.147 ± 0.014 ratio units, n = 13, $P > 0.05$) (see DISCUSSION). The effect of short and long hyposmotic challenge on fractional SR Ca\(^{2+}\) release (the ratio of electrically

![Fig. 3. Effects of the Cl⁻ current activated by swelling ($I_{Cl,Swell}$) inhibitor DIDS on the action potential in isos- and hyposmotic conditions. Representative traces showing the effects of 50 µM DIDS on action potential in isosmotic conditions (A) and after 6.5-min exposure to hyposmotic solution (B). *DIDS had no effect on action potentials recorded in isosmotic conditions (traces are superimposed) but partially reversed the shortening of the APD seen in hyposmotic conditions.](http://ajpheart.physiology.org/)
induced transient amplitude to caffeine-induced transient amplitude) is shown in Fig. 5B. After short hyposmotic challenge, fractional release was significantly increased ($P < 0.01$) by 10%. Conversely, at 10 min of hyposmotic challenge, fractional release was significantly reduced ($P < 0.05$) by 20%.

To further investigate the effect of long exposure to hyposmotic solution on SR function, we examined the effect of hyposmotic challenge on fractional SR Ca$^{2+}$ release. Fractional release represents the ratio of electrically induced transients to caffeine-induced transients. *$P < 0.05$; **$P < 0.01$ compared with isosmotic conditions (paired Student’s t-test).

Fig. 6. Effects of hyposmotic swelling on basal- and isoprenaline (Iso)-stimulated phosphorylation of phospholamban (pPLB) at Ser$^{16}$ and Thr$^{17}$. Cells were exposed to either isosmotic solution (open bars) or hyposmotic solution (hatched bars) for 5 min in the absence of Iso (Basal) or the presence of 1 µM Iso. Bars represent the means ± SE of 8 observations.

DISCUSSION

We report the effects of hyposmotic cell swelling on mammalian cardiac E-C coupling. Our experiments were carried out with isosmotic and hyposmotic solutions, which were isoionic. Some studies looking at the effects of cell swelling used simple dilution to lower the osmolarity of the perfusing solution. The protocol that we have employed in this study ensures that the effects we see when changing between isosmotic and hyposmotic solutions are due to the difference in osmolarity and not to a change in concentration of current-carrying ions.

Exchanging standard physiological solution for isosmotic solution (made by decreasing the NaCl concentration to 57 mM NaCl and adding 113 mM sucrose) resulted in an increase in APD$_{90}$, $I_{Ca,L}$ amplitude, diastolic [Ca$^{2+}$], transient amplitude, and contraction. The observed effects of switching to isosmotic solution are consistent with enhanced entry (28) or reduced extrusion of Ca$^{2+}$ via the Na$^+$-Ca$^{2+}$ exchanger during diastole, as a result of the lower external sodium concentration. This would increase the Ca$^{2+}$ load of the SR and thus the amplitude of the calcium transient and contraction. The larger calcium transient would be expected to induce a larger (Ca$^{2+}$ extruding) inward Na$^+$-Ca$^{2+}$ exchange current ($I_{NaCa}$) during the action potential, thereby accounting for the increase in APD as a result of prolongation of repolarization (27). The observed increase in the amplitude of $I_{Ca,L}$ could be explained by a facilitation of $I_{Ca,L}$ by the increase in [Ca$^{2+}$], as observed previously in cardiac myocytes from both the frog and guinea pig (14).

Although we saw no significant change in cell dimension at 1 min after hyposmotic challenge, our results after long hyposmotic challenge support those found by others (4, 25), in that hyposmotic swelling is characterized by a marked increase in the radial diameter of the cell with only a small increase in cell length. This...
response reinforces the difference between cell swelling and other forms of cell stretch. As well as the concurrent dilution of intracellular contents, the major effect of swelling is an increase in cell width, whereas axial stretch increases the length of the cell. The magnitude of the increase in cell volume that we recorded is similar to that seen by Suleymanian et al. (32) after 10-min exposure of rabbit ventricular myocytes to an hyposmotic solution of similar osmolality to that used in the present study.

When isoosmotic solution was replaced by hyposmotic solution, we observed, in the first minute of exposure, a significant increase in APD, [Ca\(^{2+}\)]\(_i\), transient amplitude, and contraction and a significant reduction in the amplitude of I\(_{\text{Ca,L}}\). At this time point, we saw no change in resting membrane potential or in diastolic [Ca\(^{2+}\)]\(_i\). In experiments performed to investigate SR function after short hyposmotic challenge, caffeine-induced fura 2 transients (indicative of SR Ca\(^{2+}\) load) were identical in isoosmotic and hyposmotic conditions. However, fractional release of Ca\(^{2+}\) from the SR was significantly increased under these conditions.

Fractional Ca\(^{2+}\) release is dependent on both the load of the SR and the trigger (2); therefore, after short hyposmotic challenge we would expect a reduction in fractional release, given that the I\(_{\text{Ca,L}}\) amplitude is decreased in the absence of an increase in SR load. However, at this time point, fractional release was increased. The gain of Ca\(^{2+}\) release, which is described by the relationship between the amplitude of I\(_{\text{Ca,L}}\) and the amplitude of the [Ca\(^{2+}\)]\(_i\) transient (38), is greater after hyposmotic exposure. Therefore, it appears that the coupling between Ca\(^{2+}\) entry and Ca\(^{2+}\) release is enhanced. This could account for the observed increase in [Ca\(^{2+}\)]\(_i\) transient amplitude and contraction. Another mechanism that could account for the change in contractility observed at this time point, consistent with the observed decrease in I\(_{\text{Ca,L}}\), is a reduction in the inactivation of Ca\(^{2+}\) release from the SR, perhaps as a result of a decrease in [Ca\(^{2+}\)] in the environment of the ryanodine receptor.

In the rat, it has been shown that the time course of the repolarization phase of the action potential is influenced by inward current generated by Na\(^+-\text{Ca}^{2+}\) exchange (27). Therefore the increase in the [Ca\(^{2+}\)]\(_i\) transient after short hyposmotic challenge could account for the observed increase in APD. In guinea pig ventricular myocytes, it has been suggested that inhibition of I\(_{\text{Kr}}\) may account for the lengthening of the APD seen within 1 min of hyposmotic challenge (24, 35). Although this may be involved in the lengthening of APD in the present study, there is evidence that I\(_{\text{Kr}}\) is less important in the rat than the guinea pig (18).

After a longer exposure to the hyposmotic solution (7–10 min), APD, I\(_{\text{Ca,L}}\) amplitude, fura 2 transient amplitude, and contraction were depressed compared with measurements made in isoosmotic solution. A depolarization of the resting membrane potential was observed at this time point. I\(_{\text{Ca,L}}\) was also significantly depressed compared with measurements made after short hyposmotic challenge. The shortening of APD after long hyposmotic challenge has been ascribed in part to a delayed activation of I\(_{\text{Cl,swell}}\) in guinea pig ventricular myocytes, which may override the more immediate effect of swelling on I\(_{\text{Kr}}\) in this species (35). The results of the present study are consistent with the involvement of I\(_{\text{Cl,swell}}\) in the shortening of the APD after long hyposmotic challenge, because we observed a partial reversal of the abbreviation of the APD at this time point in the presence of the I\(_{\text{Cl,swell}}\) inhibitor DIDS. The marked reduction in I\(_{\text{Ca,L}}\) seen after long hyposmotic challenge could also contribute to the reduction in APD both directly and indirectly through an effect on inward I\(_{\text{NaCa}}\).

Electrophysiological measurements in this study were made in the whole cell configuration of the patch-clamp technique, whereas [Ca\(^{2+}\)]\(_i\), SR function, and contractility were assessed in cells with an intact sarcolemma. Although the majority of studies on the electrophysiological effects of swelling have been carried out using the whole cell patch-clamp technique, caution should be applied when extrapolating findings from such studies to those performed in cells with intact membranes. The extent and time course of changes in cell volume and in the intracellular milieu that occur during swelling may be different from those observed in the intact cell because of the large reservoir of solution within the patch pipette.

The biphasic effect of hyposmotic swelling on APD that we observed in the present study has been reported in guinea pig ventricular myocytes using the whole cell patch-clamp technique (35). In that study, the lengthening of APD reached a peak at 45 s and declined to 79% of control values at 3 min after perfusion with hyposmotic solution. Biphasic changes in APD have also been reported in a recent perforated patch-clamp study using rabbit myocytes (22). The biphasic changes in APD we observed are also consistent with changes in contraction and [Ca\(^{2+}\)]\(_i\), recorded from cells with intact membranes. Therefore, we do not feel that the use of the whole cell patch-clamp configuration has influenced the qualitative response of the APD to swelling.

This is the first time that a reduction in I\(_{\text{Ca,L}}\) has been observed during both the early and late phases of swelling of cardiac cells (see Ref. 6). We did not observe continuous rundown of I\(_{\text{Ca,L}}\) in isoosmotic conditions, so we do not attribute the changes in I\(_{\text{Ca,L}}\) observed after hyposmotic challenge to cell inflation or the intracellular dialysis associated with establishment of the whole cell configuration. No consensus as to the effect of swelling on I\(_{\text{Ca,L}}\) has emerged from studies carried out using the whole cell patch-clamp technique (6). However, using the perforated patch-clamp technique, Li and Baumgarten (22) have recently observed a small transient increase in I\(_{\text{Ca,L}}\) after 2 min of hyposmotic challenge, which was followed by a marked depression of I\(_{\text{Ca,L}}\) at 10 min after hyposmotic challenge. This observation raises the possibility that in cells with intact membranes, I\(_{\text{Ca,L}}\) may initially increase in response to swelling and that such an increase in Ca\(^{2+}\) influx could contribute to the increase in contraction,
[Ca\(^{2+}\)]_i, and prolongation of the APD seen at this time point.

One explanation for the marked reduction in \(I_{\text{Ca,L}}\) after 10 min of swelling is a loss of t-tubules after a long exposure to hyposmotic solution, as seen after formamide-induced osmotic shock (16). Evidence suggests that the L-type Ca\(^{2+}\) channel is concentrated in the t-tubular, rather than the surface, sarcolemma (16, 21) and that t-tubules are a particularly important site for the coupling of Ca\(^{2+}\) entry through the L-type Ca\(^{2+}\) channel and Ca\(^{2+}\) release from the SR (28). However, in the present study, data obtained using the lipophilic voltage-sensitive indicator di-8-ANNEPS failed to demonstrate a loss of t-tubules after 10 min exposure to hyposmotic solution. We did see a significant increase in t-tubular space after swelling, but the magnitude of the increase was consistent with the observed increase in cell length at this time point, suggesting that it does not arise as a result of “pinching off” of t-tubules from the sarcolemma. The surface scans of cells at this time point, however, show membrane blebbing, which may potentially affect surface sarcolemmal Ca\(^{2+}\) channels. However, this cannot entirely account for the effects of long hyposmotic swelling on \(I_{\text{Ca,L}}\) that we observed, because the majority of L-type Ca\(^{2+}\) channels are concentrated in the t-tubules (16).

Although no study has examined the effects of short (1 min) hyposmotic challenge on [Ca\(^{2+}\)]_i and contraction, there are some data concerning the effects of longer hyposmotic challenge on these parameters. After 4 min exposure of rabbit ventricular myocytes to hyposmotic solution, an increase in cell shortening has been observed (22). The same study showed a subsequent decrease in contractility at 20 min after hyposmotic challenge. Lado et al. (19) reported a depression of contractility associated with a decrease in intracellular Ca\(^{2+}\) activity in sheep ventricular muscle exposed to a solution of 75% tonicity for \(\sim 5\) min. Measurements of Ca\(^{2+}\) activity were made with a Ca\(^{2+}\)-sensitive microelectrode and did not give the resolution of diastolic and systolic [Ca\(^{2+}\)]_i, but gave an average level of [Ca\(^{2+}\)]_i. The decrease in intracellular Ca\(^{2+}\) activity was thought to be secondary to a decrease in intracellular Na\(^+\) activity, which has also been observed in single rabbit ventricular myocytes after sustained hyposmotic challenge (37). The decrease in intracellular Na\(^+\) activity was ascribed to stimulation of the Na\(^+\)-K\(^-\) ATPase through an increase in the affinity of the pump for Na\(^+\), perhaps through a swelling-induced change in the kinase/phosphatase balance (3). In terms of contractility, it is possible that an increase in interfilament spacing during prolonged exposure to hyposmotic solution could contribute to the observed depression in contractility by reducing myofilament Ca\(^{2+}\) sensitivity (12).

Data obtained by Lado et al. (19) and Whalley et al. (37) suggest that sustained hyposmotic challenge reduces intracellular Na\(^+\) activity. This would tend to reduce the Ca\(^{2+}\) load of the SR via effects on the Na\(^+\)-Ca\(^{2+}\) exchanger (30). Experiments were performed to investigate the effect of long hyposmotic challenge on SR function. In the present study, caffeine-induced transients were smaller (although the difference was not significant) after long hyposmotic challenge compared with those recorded in isosmotic conditions. Given the potential effect of 10 min of swelling on the relationship between Ca\(^{2+}\) and fura 2 fluorescence, and the evidence for a decrease in intracellular Na\(^+\) activity (19, 37), it is possible that a decrease in the Ca\(^{2+}\) load of the SR does occur after prolonged hyposmotic challenge.

We also examined the effect of swelling on the phosphorylation of the SR protein phospholamban at Ser\(^{16}\) or Thr\(^{17}\). The phosphorylation status of phospholamban controls the activity of the SR Ca\(^{2+}\)-ATPase and is therefore related to the Ca\(^{2+}\) load of the SR (15). A decrease in phosphorylation of phospholamban at either site would decrease Ca\(^{2+}\) uptake by the SR. Under basal conditions, we saw no significant effect of swelling on phosphorylation on phospholamban at either site. Furthermore, we looked at the effect of the \(\beta\)-agonist isoproterenol, which increases phosphorylation at Ser\(^{16}\) and Thr\(^{17}\) through the action of protein kinase A and Ca\(^{2+}\)-calmodulin kinase, respectively (8). Isoproterenol-stimulated phosphorylation, however, was similar in isosmotic conditions and after exposure to hyposmotic solution. Although there is evidence that swelling may alter the balance between kinase and phosphatase activity (3), it appears that a change in Ca\(^{2+}\) uptake by the SR as a result of an action on the phosphorylation status of phospholamban cannot account for the effects of swelling on Ca\(^{2+}\) and contractility.

Long exposure to hyposmotic solution resulted in a significant depression of fractional SR Ca\(^{2+}\) release. Because fractional release of Ca\(^{2+}\) from the SR depends on the size of \(I_{\text{Ca,L}}\), which triggers Ca\(^{2+}\) release (2), at this time point we may conclude that the reduction in fractional SR Ca\(^{2+}\) release arises as a result of reduced trigger. A possible reduction in SR Ca\(^{2+}\) load might also contribute to the depression of fractional SR Ca\(^{2+}\) release. The fall in Ca\(^{2+}\) transient amplitude and contractility following long hypo-osmotic swelling could be ascribed to the depression of fractional SR Ca\(^{2+}\) release at this time point.

In conclusion, in the present study we have demonstrated, for the first time, a simultaneous biphasic modulation of contractility and [Ca\(^{2+}\)]_i that is consistent with biphasic changes in APD after hyposmotic challenge. Initially, a positive inotropic effect is observed, but later, contractility is depressed. Both phases are associated with a decrease in the amplitude of \(I_{\text{Ca,L}}\); this decrease is greater after sustained hyposmotic challenge. The positive inotropic effects of short hyposmotic challenge may be due to increased efficiency of coupling between Ca\(^{2+}\) entry and SR Ca\(^{2+}\) release or to a reduction in the inactivation of Ca\(^{2+}\) release from the SR. After long hyposmotic challenge, there is no mechanism to compensate for the marked reduction in \(I_{\text{Ca,L}}\); coupling between Ca\(^{2+}\) entry and release is not enhanced, and contraction falls. The mechanism(s) that may lead to the initial increase in
efficiency of E-C coupling and to the fall in $I_{Ca,L}$ after hyposmotic challenge have yet to be established.

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