Membrane currents underlying the modified electrical activity of guinea-pig ventricular myocytes exposed to hyperosmotic solution

Toshitsugu Ogura, Yongdong You and Terence F. McDonald *

Department of Physiology and Biophysics, Dalhousie University, Halifax, Nova Scotia, Canada B3H 4H7

1. Guinea-pig ventricular myocytes were superfused with hyperosmotic (sucrose) Tyrode solution (1.2–2.8 times \((T)\) normal osmolality) for up to 40 min. Action potentials were recorded with microelectrodes, and membrane currents with the perforated- or ruptured-patch technique.

2. Hyperosmotic treatment for 20 min shrunk cell volume and hyperpolarized the membrane. Moderate \((1.2–1.5 \times T)\) treatment caused biphasic changes in action potential configuration (rapid minor shortening quickly followed by lengthening to a stable 110\% control duration). Severe \((2.2–2.8 \times T)\) treatment caused triphasic changes (marked early shortening, strong rebound lengthening and subsequent pronounced shortening). At peak lengthening (6–10 min) action potentials (165\% control duration) had a hump near −30 mV and slowed terminal repolarization.

3. In accordance with previous studies, hyperosmotic solution inhibited the delayed rectifier \(K^+\) current, and enhanced the outward \(Na^+-Ca^{2+}\) exchange current \((I_{NaCa})\) at plateau potentials. A novel finding was that hyperosmolality reduced the amplitude of \(L\)-type \(Ca^{2+}\) current \((I_{Ca,L})\) and slowed its rate of inactivation. Experiments on myocytes loaded with indo-1 suggest that the reduction in \(I_{Ca,L}\) is due to a rapid elevation of \([Ca^{2+}]\).

4. When impaled myocytes were preloaded with EGTA, severe hyperosmotic treatment induced a rapid monotonic shortening of the action potential to a stable 20\% of control duration. Addition of external \(K^+\) quickly nulled the hyperpolarization and slowly lengthened the action potential.

5. The results suggest that modified electrical activity in osmotically shrunken myocytes is primarily caused by increases in \([K^+]\), \([Na^+]\), and \([Ca^{2+}]\): (i) elevated \([K^+]\) hyperpolarizes the membrane (which may contribute to increased \([Na^+]\)); (ii) elevated \([Na^+]\) shortens all phases of the action potential (increased outward-directed \(I_{NaCa}\)); and (iii) elevated \([Ca^{2+}]\) has antagonistic plateau shortening (inhibition of inward \(I_{Ca,L}\)) and plateau lengthening (reduced outward \(I_{NaCa}\)) influences, as well as a strong subplateau lengthening effect (enhanced inward \(I_{NaCa}\)).

External hyperosmotic solution pulls water out of cells, and the resultant passive and volume-transduced changes in intracellular osmolyte concentrations and membrane transport-protein activity (Hoffmann & Simonsen, 1989; Sarkadi & Parker, 1991) are certain to cause significant modifications of electrical activity in excitable cells. In the first investigation in this area, Adrian (1956) found that hyperosmotic conditions increased \([K^+]\) and hyperpolarized the resting membrane of frog skeletal muscle fibres. Subsequent work by Page & Storm (1966) and Fozzard & Lee (1976) demonstrated that hyperosmotic sucrose solution increased \([K^+]\) and hyperpolarized the membrane in isolated cardiac tissue. Although these and other studies (Hermesmeyer, Rulon & Sperelakis, 1972; Bailey, 1981; Ehara & Hasegawa, 1983; Lado, Sheu & Fozzard, 1984; Whalley, Hool, Ten Eick & Rasmussen, 1993) have clearly documented the effects of hyperosmotic solution on the cardiac resting potential, the effects on action potential configuration are less clear. A shortening of action potential duration in multicellular cardiac preparations has been reported (Hermesmeyer et al 1972), but so has a prolongation (Bailey, 1981; Ehara & Hasegawa, 1983). As far as we are aware, there have been

* To whom correspondence should be addressed.
no studies on the effects of hyperosmotic solution on action potentials in isolated cardiomyocytes.

A straightforward prediction of the changes in action potential configuration likely to occur during hyperosmotic stress is precluded by the multiplicity of intertwining factors that need to be considered. The first of these is that, aside from an elevation of $[K^+]_i$, one can expect elevations of $[Na^+]_i$ and $[Ca^{2+}]_i$ (Lado et al. 1984; Allen & Smith, 1987; Whalley et al. 1993); at the very least, these elevations will affect the action potential by modifying the driving forces on ionic currents. Secondly, the initial resetting of intracellular ion concentrations caused by the rapid exodus of cell water is unlikely to be a stable situation; additional changes over the longer term may ensue from modifications of electro-neutral and electric transporter activity (Drewnowska & Baumgarten, 1991; Rasmussen, Davis & Lieberman, 1993; Whalley, Hemsworth & Rasmussen, 1994). Thirdly, whether as a result of altered intracellular ion concentrations or volume-transduced mechanisms, the modified activity of a number of electrogenic transporters may have an impact on the action potential. In this regard, recent patch-clamp studies on heart cells indicate that hyperosmotic superfusate inhibits Na$^+$/K$^+$ pumping (Whalley et al. 1993; Sasaki, Mitsuiye, Wang & Noma, 1994) and stimulates Na$^+$/Ca$^{2+}$ exchange (Wright, Rees, Vandenberg, Twist & Powell, 1995). Fourthly, hyperosmotic conditions can affect ion channels in a manner that appears to be independent of changes in intracellular ion concentrations. For example, hyperosmotic superfusates strongly inhibited the delayed rectifier K$^+$ current ($I_K$) in guinea-pig ventricular myocytes (Sasaki et al. 1994), and depressed basal Cl$^-$ current ($I_{Cl}$) in rabbit atrial myocytes (Duan, Fermini & Natel, 1995).

In the present study, we have recorded action potentials and whole-cell membrane currents from guinea-pig ventricular myocytes superfused with solutions that, by addition of sucrose, were up to 2-8 times ($T$) the osmolality of control 1$T$ solutions. The action potentials were generally recorded with high-resistance microelectrodes to minimize disturbance of the intracellular milieu; however, in some experiments they were recorded using lower-resistance microelectrodes filled with a 0.3 M EGTA solution to buffer cytoplasmic Ca$^{2+}$. In the voltage-clamp studies, membrane currents were recorded by application of either the nystatin perforated-patch method or the standard ruptured-patch method. The perforated-patch method minimized the washout of cytoplasmic constituents, and allowed hyperosmotically induced changes in intracellular Ca$^{2+}$ to proceed in a manner resembling that in the impaled myocytes. Conversely, the ruptured-patch method permitted a degree of control over intracellular Na$^+$, Ca$^{2+}$ and K$^+$ by varying Na$^+$, Ca$^{2+}$/EGTA and K$^+$/Ca$^{2+}$ in the pipette-filling solution. These manipulations allowed closer examination of changes in membrane current components and the factors responsible for them. Additional information useful in the interpretation of the results was obtained from measurements of myocyte shrinkage and cytoplasmic [Ca$^{2+}$]$_i$ (indo-1 method).

**METHODS**

**Preparation of myocytes**

All procedures were performed in accordance with national and local regulations on animal experimentation. Male guinea-pigs (250-350 g) were killed by cervical dislocation. Hearts were excised, mounted on a Langenfeld column and perfused (37 °C) for 10-15 min with disociating solution that contained collagenase (0.08-0.12 mg ml$^{-1}$; Yakult Pharmaceutical Co., Tokyo, Japan). The cells were dispersed in a high-K$^+$ nutrient-supplemented storage solution and stored at 22 °C. For the experiments, a few drops of the cell suspension were placed in a 0.3 ml perfusion chamber mounted on an inverted microscope stage. After the cells had settled to the bottom, the chamber was perfused with Tyrode solution (26 °C) at ~2 ml min$^{-1}$. Only rod-shaped quiescent cells with smooth contours were selected for study.

**External solutions**

Ca$^{2+}$-free dissociating solution contained (mm): NaCl, 120; KCI, 4.6; MgCl$_2$, 1.2; taurnine, 20; glucose, 20; and Hepes, 5 (pH 7.4 with NaOH). Storage solution contained (mm): KOH, 80; KCl, 30; KH$_2$PO$_4$, 30; MgSO$_4$, 6; glutamic acid, 50; taurnine, 20; glucose, 20; EGTA, 0.5; and Hepes, 10 (pH 7.4 with KOH).

Tyrode solution contained (mm): NaCl, 138.5; KCl, 4.6; CaCl$_2$, 2.45; MgCl$_2$, 1.2; glucose, 10; and Hepes, 5 (pH 7.4 with NaOH). Osmolality measured by freezing-point depression (a osm, Precision Systems Inc., SUlbury, MA, USA) was 293 ± 2 mosmol (kg H$_2$O)$^{-1}$ (mean ± s.e.m., n = 12). Hyperosmotic 1.2 T, 1.5 T, 2.2 T and 2.8 T Tyrode solutions were made by inclusion of 50, 100, 150 and 450 mm sucrose, respectively. Na$^+$/free Tyrode solution was made by equimolar substitution of tetramethylammonium for Na$^+$.

**Electrophysiology and pipette-filling solutions**

Membrane potentials were recorded with 3 M KCl-filled micro-electrodes (resistance, 30-80 M$\Omega$) connected to a high-input impedance amplifier (model 8100, Dagan Corp., Minneapolis, MN, USA) via an Ag–AgCl half-cell. A down-stream-positioned 3 M KCl–Ag–AgCl unit with a frittered glass junction was used as a reference electrode. Unless otherwise noted, myocytes were stimulated at 1 Hz with 1 ms suprathreshold current pulses passed through the microelectrode using an active bridge circuit. In some experiments, microelectrodes with relatively low resistance (25-50 M$\Omega$) were filled with 0.3 M EGTA–0.9 M KOH solution (pH 7.3 with KOH).

Myocytes were voltage clamped using either the nystatin perforated-patch method or the standard ruptured-patch method. Recording pipettes were fabricated from thick-walled borosilicate glass capillaries (H15-10137, Jencons Scientific Ltd, Bedfordshire, UK) and had resistances of 2-3 M$\Omega$ when filled with solution. The mean osmolalities of the pipette-filling solutions ranged between 300 and 308 mosmol (kg H$_2$O)$^{-1}$ with an s.e.m. of 2–4 mosmol (kg H$_2$O)$^{-1}$ on ten to twelve measurements. Liquid junction potentials between external and pipette-filling solutions were offset before the pipette touched the cell. Membrane currents were recorded with an EPC-7 amplifier (List Electronic). The electrical signals were stored on videotape through a pulse-code modulator (PCM-2-B, Medical Systems Corp., Greensville, NY, USA) for off-line computer analysis (sampling frequency, 5-10 kHz).

**Perforated patch.** In the perforated-patch experiments, the series resistance ($R_s$) was monitored after seal formation. $R_s$ declined to 8–25 M$\Omega$ within 10–20 min of seal formation and experiments were initiated when $R_s$ was stable over a 10 min period. $R_s$ compensation was used in most of the experiments such that uncompensated $R_s$ was reduced below 10 M$\Omega$ (typically 2–6 M$\Omega$).
Pipettes were filled with either a K⁺-rich or Cs⁺-rich solution (Table 1). Nystatin (Sigma) was added to the pipette-filling solution from a stock solution (100 mg ml⁻¹ in dimethyl sulphoxide) to give a final concentration of 50—100 µg ml⁻¹. The nystatin stock solution was freshly prepared and used within 2 h.

Ruptured patch. Recording pipettes in the ruptured-patch experiments were filled with either (i) 10 mM Na⁺ pCa 7 K⁺ solution, or (ii) Na⁺-free pCa 9 Cs⁺ solution (Table 1). Rₛ ranged between 5 and 9 MΩ and was compensated by 60—80 %.

Myocyte volume
Cell images were recorded on videotape using a television system (series 67 camera system; Dage-MTI Inc., Michigan City, IN, USA), and cell width and length were measured from replayed images (Ogura, Shuba & McDonald, 1995); the measurements were reproducible to <1 %. Relative cell volume was calculated as length x (width)² on the assumption that there are proportional changes in cell width and thickness under anisosmotic conditions (Roos, 1986; Drewnowska & Baumgarten, 1991; Duan et al. 1995; Ogura et al. 1995).

Fluorescence measurements
[Ca²⁺], was measured using a PTI-Deltascan-4000 ratio-fluorescence system (Photon Technology International Inc., South Brunswick, NJ, USA) as described for fura-2 by You, Pelzer & Pelzer (1994). Unpatched myocytes were loaded with indo-1 by adding a mixture of 1 mM indo-1 AM (acetoxymethyl ester form, 5 µl; Molecular Probes) and 25 % (w/w) Pluronic F-127 (1·3 µl; Calbiochem) (both

Figure 1. Effects of 2·2 T hyperosmotic solution on resting potential and dimensions of myocytes impaled with high-resistance microelectrodes
A, time course of the change in resting potential (ΔRP) of myocytes stimulated at 1 Hz. B, time course of ΔRP in quiescent myocytes. C, changes in the dimensions of the myocytes studied in B. Cell length and width are expressed as percentages of control values and relative cell volume was calculated as length x (width)². Values are presented as mean ± s.e.m. (n = 7). All data during hyperosmotic treatment are significantly different (P < 0·05) from control values.

Table 1. Composition of pipette-filling solutions (mM)

| Potassium | Caesium | KCl | CaCl₂ | MgCl₂ | MgSO₄ | CaCl₂ | Na₂ATP | MgATP | EGTA | Heps |
|-----------|---------|-----|-------|-------|-------|-------|--------|--------|------|------|-----|
| Perforated-patch recording | | | | | | | | | | | |
| Na⁺ free, K⁺ rich | 30 | — | 110 | — | 5 | — | — | — | — | 5 |
| Na⁺ free, Cs⁺ rich | — | 30 | — | 110 | 5 | — | — | — | — | 5 |
| Ruptured-patch recording | | | | | | | | | | | |
| 10 mM Na⁺, K⁺ rich, pCa 7 | 40 | — | 100 | — | 5 | 2 | 5 | — | 5 | 5 |
| Na⁺ free, Cs⁺ rich, pCa 9 | — | 40 | — | 105 | 2 | — | — | 4 | 0·2 | 10 |

pH was adjusted to 7·2 with KOH or CsOH. Assuming Ca²⁺ contamination to be ~1 µM, pCa was estimated using a program provided by Fabiato & Fabiato (1979), with the correction of Tsien & Rink (1980).
in dimethyl sulphoxide) to 1 ml cell suspension for > 30 min at ~22 °C. Ruptured-patch myocytes were loaded with indo-1 by dialysis with pipette-filling solution that contained 50 ìM indo-1 (pentapotassium salt; Molecular Probes). The excitation was set at 345 nm by a monochromator (bandpass 10 nm) and the emission was split by a dichroic cube assembly, comprising a dichroic mirror (455 nm) and two bandpass filters (405/10 nm and 485/10 nm). After background correction, the ratio \( R \) of emission fluorescence at 405 and 485 nm was converted to \([Ca^{2+}]_e\) according to:

\[
[Ca^{2+}]_e = K_D \beta (R_{\text{min}} - R) / (R_{\text{max}} - R),
\]

where \( R_{\text{min}} \) and \( R_{\text{max}} \) denote \( R \) obtained in Ca\(^{2+}\)-free and Ca\(^{2+}\)-saturated indo-1 samples, respectively, and \( \beta \) is the fluorescence intensity ratio of the Ca\(^{2+}\)-free and Ca\(^{2+}\)-saturated samples at 485 nm. \( K_D \) (392 nM), \( \beta \) (1.934), \( R_{\text{min}} \) (0.147) and \( R_{\text{max}} \) (1.296) were determined as described by You et al. (1994).

**Statistics**

Results are expressed as means ± s.e.m. \( n \) represents the number of experiments. Single comparisons were made using Student's \( t \) test. Analysis of variance followed by Bonferroni's test was used for multiple comparisons including analysis of repeated measurements. Differences were considered significant when \( P < 0.05 \).

**RESULTS**

Changes in the size, resting potential and action potential configuration of impaled myocytes superfused with hyperosmotic solution

Hyperosmotic 2·2 \( T \) solution hyperpolarized the resting membrane of myocytes impaled with high-resistance microelectrodes. The hyperpolarization (ARP) in 1 Hz-stimulated myocytes reached \(-12 ± 0·6 \text{ mV} \ (n = 7)\) after 2 min and was relatively constant thereafter (Fig. 1A). A similar-sized hyperpolarization was recorded from quiescent myocytes (Fig. 1B). The probable cause of these hyperpolarizations was an increase in \([K^+])\, due to osmotically induced loss of water from the cells. As shown in Fig. 1C, the time course of volume shrinkage in quiescent microelectrode-impaled myocytes was similar to that of ARP. After 10 min 2·2 \( T \) treatment, relative myocyte volume was 67 ± 1% and ARP was \(-12 ± 0·5 \text{ mV} \ (n = 7)\).

Hyperosmotic 1·5, 2·2 and 2·8 \( T \) solution caused multiphasic time-dependent changes in the action potential duration of myocytes stimulated at 1 Hz (Fig. 2). The earliest was a

![Figure 2. Effects of hyperosmotic solutions on action potential configuration](image_url)
shortening that was mainly due to a depressed plateau phase. This was followed by a lengthening which, especially with 2·8T treatment, featured a humped repolarization near −30 mV and slowed terminal repolarization (Fig. 2C). Subsequent shortening during severe hyperosmotic challenge resulted in action potentials that had very short plateaus and slow terminal repolarization phases that began near −50 mV (e.g. Fig. 2C, 20 min).

A summary of the changes in action potential parameters measured during 20 min exposures to hyperosmotic solution is shown in Fig. 3. The steady-state values for ΔRP were −2 ± 0·3 mV (1·2T, n = 4), −7 ± 0·5 mV (1·5T, n = 5), −12 ± 0·6 mV (2·2T; n = 7) and −16 ± 1 mV (2·8T, n = 8) (Fig. 3A). Action potential overshoot (control, 36 ± 1 mV; n = 24) declined in an osmolality-dependent manner, with the largest steady-state reduction being 16 ± 3 mV in 2·8T solution (Fig. 3B). Both the initial shortenings and rebound lengthenings of the action potential (measured at two levels, +10 mV (action potential duration, APD+10) and 90% repolarization (APD90)) were relatively small during 1·2–1·5T treatment, and durations measured after 20 min were moderately longer than control (e.g. APD90 110 ± 3% control (n = 4) and 111 ± 2% (n = 5), 1·2T and 1·5T solutions, respectively). Stronger hyperosmotic challenges caused pronounced initial shortenings, rebound lengthenings and secondary shortenings (e.g. 2·8T (n = 8)). APD90 of 63 ± 5% control after 1 min, 165 ± 16% after 8 min and 53 ± 9% after 20 min). Compared with these changes in APD90 during 2·8T treatment, the initial shortening of APD90 (to 40 ± 6% control at 1 min) was more severe, the rebound lengthening weaker (to 49 ± 6% at 6 min), and the subsequent shortening more pronounced (to 7 ± 2% after 20 min). In three of eight myocytes exposed to 2·2–2·8T solution, there was a rapid progressive collapse of the plateau, and early appearance of a post-plateau 'hump' (see Fig. 2C, lower traces); data from these three other myocytes that depolarized and hypercontracted during the 20 min test period were excluded from the foregoing analysis.

A number of the myocytes studied above were allowed to recover for 20 min in 1T control solution. Recovery of resting potential was complete (within 2 mV of pre-challenge value) in all myocytes, but recovery of APD+10 and APD90 depended on the severity of the hyperosmotic treatment. The APD+10 and APD90 values were 99 ± 9 and 102 ± 4% of 1T control after recovery from 1·2–1·5T treatment (n = 7), 78 ± 19 and 98 ± 5% after recovery from 2·2T treatment (n = 6) and 33 ± 7 and 60 ± 4% after recovery from 2·8T treatment (n = 3).

The changes in action potential configuration induced by hyperosmotic solution are taken up again in later sections of the Results. At this point, it is useful to present the results

Figure 3. Changes in action potential parameters during exposures to hyperosmotic solutions
ΔRP (A) and action potential overshoot (B) were measured after 20 min treatment of impaled myocytes with hyperosmotic solution. The resting potential prior to treatment was −90 ± 0·4 mV (n = 24 myocytes). C and D, time course of changes in APD+10 and APD90, respectively. Values are presented as means ± s.e.m. (n = 4, 5, 7 and 8 myocytes for 1·2T, 1·5T, 2·2T and 2·8T solutions, respectively); * P < 0·05 versus control.
of voltage-clamp experiments that provide direction on the underlying causes of these changes.

**Membrane currents in perforated-patch myocytes superfused with hyperosmotic solution**

Perforated-patch myocytes (Na⁺-free K⁺ pipette solution) were held at −90 mV, depolarized to −40 mV for 150 ms to inactivate Na⁺ current (I_{Na}) and any T-type Ca²⁺ current, and then depolarized or hyperpolarized with 200 ms test pulses applied at 0.2 Hz. Figure 4 shows the results of exposing a myocyte to 1.5T solution for 8 min. On test depolarizations to 0 and +40 mV, the peak of the inward-going transient L-type Ca²⁺ current (I_{Ca,L}) was depressed by approximately 30%, the bi-exponential time course of decay of the inward transient was slowed and the amplitude of the end-of-pulse outward current was reduced (Fig. 4A and B). I_{Ca,L} amplitude was estimated as inward peak minus end-of-pulse current (ΔI) and the current–voltage.

![Figure 4](image)

**Figure 4. Effects of 1.5T hyperosmotic solution on membrane currents in a perforated-patch myocyte**

The pipette was filled with K⁺-rich solution and the myocyte was held at −90 mV. Test pulses applied to various potentials for 200 ms at 0.2 Hz were preceded by 150 ms prepulses to −40 mV. A, records obtained on pulses to 0 and +40 mV before and 8 min after exposure to 1.5T solution. The leading edges of the records show the currents at prepulse −40 mV. The dashed lines indicate zero-current level, and the dash-dot lines indicate prepulse current levels. B, time course of inactivation of I_{Ca,L} at 0 mV. The fast (τ_f) and slow (τ_s) time constants were obtained by a least-squares fit of the sum of two exponential functions to the records shown in A. C, I–V relationships for I_{Ca,L} measured as ΔI (circles), currents at 50 ms post-pulse onset (diamonds), and end-of-pulse currents (triangles) before and 8 min after exposure to 1.5T solution. Details of the current measurements are shown in the inset.
(I–V) plot in Fig. 4C (circles) indicates that hyperosmotic treatment reduced it over the entire $I_{Ca,L}$–V activation range. Figure 4C also shows that: (i) current at 50 ms into depolarizing pulses (diamonds) was more inward between −20 and +20 mV; and (ii) end-of-pulse current (triangles) was more outward at potentials negative to −30 mV, but less outward at positive potentials. In agreement with the finding (Sasaki et al. 1994) that hyperosmotic stress inhibits the delayed rectifier $I_K$ in guinea-pig ventricular myocytes, the tail currents on repolarization to −40 mV were reduced by 1·5T treatment (Fig. 4A).

The results of a similar type of experiment on two perforated-patch myocytes exposed to 2·2T hyperosmotic solution are shown in Fig. 5. Each of the changes noted above was observed within a short time of switching to the 2·2T solution. Table 2 compares the effects of 5 min 1·5T treatments with those of 2·2T on $I_{Ca,L}$ amplitude (measured as ΔI) and inactivation time constants. The 1·5T solution reduced $I_{Ca,L}$ to 72 ± 7% (n = 6) of control amplitude, whereas 2·2T reduced it to 60 ± 4% (n = 5). Inactivation time constants increased to 117 ± 9 and 129 ± 18% control (not significant) with 1·5T solution, and to 128 ± 5 and

Figure 5. Effects of 2·2T hyperosmotic solution on $I_{Ca,L}$ and end-of-pulse currents in two perforated-patch myocytes

The pipettes contained K⁺-rich solution and the myocytes were held at −100 mV. Following prepulses to −40 mV, 200 ms test pulses were applied to a constant 0 mV except for sequences of test pulses just before and 3 and/or 10 min after the switch to 2·2T solution. Pulsing rate 0·2 Hz. A, current records obtained at potentials between 0 and +40 mV before and after 10 min exposure to 2·2T solution. The dashed lines indicate zero-current level. B, time courses of changes in end-of-pulse current ($I_{200}$) and $I_{Ca,L}$ measured as net inward current ($I_n$) or inward peak minus end-of-pulse current (ΔI, see A, left) from the experiment in A. C, modifications of I–V relationships after 3 and 10 min treatment with 2·2T solution. Test pulses were applied to potentials between −110 and +50 mV (10 mV increments) and current amplitudes were measured by reference to zero current. The open symbols indicate $I_{200}$ and the filled symbols indicate $I_n$ (see B above).
$155 \pm 21\% \ (P < 0.05)$ with 2·2$T$ solution. (In five experiments with Ca$^{2+}$ (rather than K$^{+}$)-filled pipettes, 1·5$T$ solution reduced $I_{\text{Ca,L}}$ to 79 ± 2% control and significantly lengthened the inactivation time constants; Table 2.)

Although membrane currents were relatively stable after the initial 3 min of 1·5$T$ treatment, this was not the case during 2·2$T$ treatment. As illustrated by the data in Fig. 5, the initial decline in $I_{\text{Ca,L}}$ was followed by a slight recovery and then a progressive decline between 6 and 11 min. During the same time period, the end-of-pulse outward current ($I_{\text{out}}$) at potentials positive to −20 mV changed from being lower than control to being several times larger. In five myocytes, the control $I_{\infty}$ at 0 mV was 0·05 ± 0·03 nA, versus 0·28 ± 0·07 nA after 10 min superfusion with 2·2$T$ solution.

### Table 2. Changes in $I_{\text{Ca,L}}$ during hyperosmotic superfusion

| Experimental conditions | $I_{\text{Ca,L}}$ (% of 1$T$ control) | Inactivation time constant $^b$ |
|-------------------------|-------------------------------------|-------------------------------|
| Patch type              | Pipette solution | n | T | Amplitude$^a$ | Fast | Slow |
| Perforated              | Ce$^{+}$ rich        | 5 | 1·5 | 79 ± 2$^*$. | 154 ± 21$^*$. | 132 ± 14$^*$. |
|                        | K$^{+}$ rich         | 6 | 1·5 | 72 ± 7$^*$. | 117 ± 9 | 129 ± 18 |
|                        | K$^{+}$ rich         | 5 | 2·2 | 69 ± 4$^*$. | 128 ± 5$^*$. | 155 ± 21$^*$. |
| Ruptured                | Ce$^{+}$ rich        | 6 | 1·5 | 72 ± 5$^*$. | 151 ± 16$^*$. | 125 ± 12$^*$. |
|                        |                      | 4 | 2·2 | 59 ± 6$^*$. | 189 ± 16$^*$. | 157 ± 16$^*$. |

$^a$I$_{\text{Ca,L}}$ amplitude on pulses from prepulse −40 to 0 mV was measured as Δ$I$ (see Fig. 5A) after 5 min hyperosmotic superfusion. Overall control (1$T$) amplitudes were −1·18 ± 0·11 nA in perforated-patch experiments ($n = 16$) and −1·28 ± 0·15 nA in ruptured-patch experiments ($n = 10$). $^b$The fast and slow time constants were obtained by a least-squares fit of the sum of two exponential functions. In perforated-patch experiments the control (1$T$) time constants were 8·2 ± 0·9 ms (fast) and 42·8 ± 3·6 ms (slow) using K$^{+}$-rich pipette solution ($n = 11$), and 8·7 ± 1·0 ms (fast) and 48·4 ± 5·5 ms (slow) using Ce$^{+}$ solution ($n = 5$), in ruptured-patch experiments (pCa 9 Ce$^{+}$-rich pipette solution) the 1$T$ values were 7·7 ± 0·8 ms (fast) and 47·9 ± 2·5 ms (slow) ($n = 10$). $^*P < 0.05$ versus control (1$T$) values.

Involvement of intracellular Ca$^{2+}$ and intracellular Na$^{+}$

Most of the experiments described below were conducted on ruptured-patch myocytes to facilitate partial buffering of cytoplasmic Ca$^{2+}$, improved control of cytoplasmic Na$^{+}$ and, in some cases, pipette loading of myocytes with indo-1. For improved resolution of $I_{\text{Ca,L}}$, we also used Ce$^{+}$- rather than K$^{+}$-filled pipettes.

Figure 6. Effects of 2·2$T$ hyperosmotic solution on membrane currents and cytoplasmic [Ca$^{2+}$] in ruptured-patch myocytes dialysed with pCa 9 Ca$^{2+}$ solution

Myocytes were held at −90 mV, depolarized to −40 mV for 150 ms and pulsed to +20 mV for 200 ms at 0·2 Hz. A, example records and time plots of end-of-pulse current at +20 mV ($I_{\text{out}}$) and peak inward current at +20 mV ($I_{\text{in}}$). B, increases in [Ca$^{2+}$] measured from myocytes pulsed as in A. Means ± s.e.m. ($n = 3$ myocytes).
The first important result was that hyperosmotic solution reduced the amplitude and slowed the inactivation of $I_{Ca,L}$ in ruptured-patch myocytes whose intracellular $Ca^{2+}$ was moderately buffered by the pCa 9 Cs⁺-rich dialysate (e.g. Fig. 6A). As summarized in Table 2, 5 min 1·5T and 2·2T treatment reduced $I_{Ca,L}$ measured as $\Delta I$ at 0 mV to 72 ± 5% (n = 6) and 59 ± 6% (n = 4), respectively, and significantly lengthened inactivation time constants (e.g. to 189 ± 16 and 157 ± 16% control with 2·2T solution). In additional experiments, we determined that the changes in $I_{Ca,L}$ were at least partially reversible upon re-introduction of control 1T solution for 5 min. $I_{Ca,L}$ amplitude recovered from 69 ± 7% (1·5T) to 91 ± 5% (n = 4), and from 63 ± 9% (2·2T) to 89 ± 3% (n = 3). In the 2·2T experiments, the fast and slow time constants recovered from 182 ± 17 to 117 ± 19% and from 162 ± 31 to 121 ± 6%, respectively.

A further observation in ruptured-patch myocytes dialysed with pCa 9 Cs⁺-rich solution was that severe hyperosmotic treatment caused complex changes in net inward current during pulses to plateau potentials. Figure 6A (right) indicates that net inward current at the time of peak $I_{Ca,L}$ ($I_{in}$) during pulses to +20 mV declined to 75% control during the first 2 min of 2·2T treatment and then more slowly to about 50% after 6–9 min. However, from about 30 ms onward into the 200 ms pulses, net inward current was larger than control due to slowed inactivation of $I_{Ca,L}$ (e.g. current at 80 ms post-pulse onset; see arrows in Fig. 6A, left).

To obtain direction on the extent of possible changes in $[Ca^{2+}]_i$ during hyperosmotic treatment, ruptured-patch myocytes were dialysed with pCa 9 Cs⁺-rich solution that contained indo-1. The myocytes were held at −90 mV, depolarized to −40 mV for 150 ms, and pulsed to +20 mV for 200 ms at 0·2 Hz. $[Ca^{2+}]_i$ increased from 37 ± 4 to 69 ± 4 nM (n = 3) during the first 3 min of 2·2T exposure and then more slowly to 87 ± 11 nM after 10 min (Fig. 6B). Since ruptured-patch (and perforated-patch) myocytes quickly shrunk to the same stable volume as unpatched myocytes (see Fig. 1C) during hyperosmotic superfusion (not shown), it seems likely that most of the early increase in $[Ca^{2+}]_i$ was due to dehydration.

The results to this point have identified some of the changes in membrane currents that may be responsible for the altered configuration of the action potential during hyperosmotic challenges. However, they do not adequately explain why, during the initial 1–2 min of strong challenges, there is such a marked shortening of the action potential plateau (e.g. Fig. 3C), nor why, at a later stage, the action potential acquires a pronounced slowed terminal repolarization phase.

Figure 7. Rapid effects of 2·2T hyperosmotic solution on membrane currents in two ruptured-patch myocytes dialysed with 10 mM Na⁺, pCa 7 K⁺ solution. The myocytes were held at −90 mV, depolarized to −40 mV for 50 ms and tested with 100 ms pulses to +10 or +30 mV at 0·2 Hz. A, example records from the myocyte pulsed to +10 mV. The dashed lines indicate zero current level. B, time plot of end-of-pulse current at +10 mV ($I_{pre}$; ○) and $I_{in}$ (●) from the myocyte in A. C, example records from the myocyte pulsed to +30 mV. D, time plot of $I_{Ca,L}$ (○) and tail current ($I_{tail}$; ▲) amplitude from the myocyte in C. Tail current amplitude was measured 10 ms after repolarization from +30 to −90 mV by reference to steady-state holding current.
One of the factors that was almost certainly different in the voltage-clamped myocytes than in the impaled myocytes was \([Na^+]_e\), i.e. the pipettes in the voltage-clamp experiments described to this point were filled with Na\(^+\)-free solution, a condition that is likely to have kept [Na\(^+\)]\(_e\) below that in the impaled myocytes. To determine whether \([Na^+]_e\) has a significant effect on changes in membrane currents, we performed experiments on ruptured-patch myocytes dialysed with a 10 m\(\Omega\) Na\(^+\) solution. This K\(^+\)-rich solution was buffered to pCa 7 to resemble the \([K^+]_e\) and \([Ca^{2+}]_e\) situation likely to prevail in pulsed perforated-patch myocytes. As shown in the two examples of Fig. 7, one of the rapid effects of 2·2\(T\) solution during 0·2 Hz pulsing from prepulse −40 mV to test potential +10 mV (Fig. 7A and B) or +30 mV (Fig. 7C and D) was a large outward shift in the test current. Since this early increase in outward current did not occur in ruptured-patch myocytes dialysed with Na\(^+\)-free solution (Fig. 6A), it suggests that 'control' of cytoplasmic Na\(^+\) by the pipette was limited and unable to prevent a shrinkage-induced elevation of [Na\(^+\)]\(_e\) (and consequently [Ca\(^{2+}\)]\(_e\)) in regions near the membrane. (See Mogul, Singer & Ten Eick (1989) and Carmeliet (1992) for in-depth discussions on 'control' and heterogeneity of [Na\(^+\)]\(_e\) in myocytes.) The rapid reduction of \(I_{Ca,L}\) (Fig. 7A–C) and emergence of large inward tail currents on repolarization to the holding potential (Fig. 7A, C and D) suggest that there was a pronounced accumulation of Ca\(^{2+}\) in these myocytes.

The lack of control over [Na\(^+\)]\(_e\) was likely to have been more serious in the perforated-patch myocytes than in the ruptured-patch myocytes due to the much higher access resistance (for discussions on importance of pipette

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**Figure 8. Effects of Ni\(^{2+}\) addition and Na\(^+\) removal on membrane currents activated by 2·2\(T\) solution in perforated-patch myocytes**

The pipettes contained K\(^+\)-rich solution, and the myocytes were held at −100 mV. Test pulses applied to +20 mV for 200 ms at 0·2 Hz were preceded by 150 ms prepulses to −40 mV. Test pulse sequences to potentials between 0 and +40 mV were applied before (1T) and after 8–9 min exposure to 2·2\(T\) solution. Thereafter, the superfuse was switched to either 2·2\(T\) plus 5 m\(\Omega\) Ni\(^{2+}\) solution (left-hand panels) or Na\(^+\)-free (0 Na) 2·2\(T\) solution (right-hand panels) for 3 min (+20 mV pulsing) and the test sequence applied once more. End-of-pulse current \(I_{200}\) was measured by reference to zero current; inward tail current \(I_{tail}\) at 20 ms repolarization was measured by reference to steady-state holding current. A, effect of Ni\(^{2+}\) on \(I_{200}\). B, effect of Ni\(^{2+}\) on the amplitude of tail currents elicited by repolarizations from test potentials between 0 and +40 mV. Example records of tail currents from 0 mV (upper traces) and +40 mV (lower traces) are shown below the plot. The dashed lines indicate zero current level, and the dash-dot lines indicate steady-state holding currents. C, effect of Na\(^+\) removal on \(I_{200}\). D, effect of Na\(^+\) removal on tail current amplitude. Example records presented as in B.
resistance see: Mogul et al. 1989; Shuba, Heßliger, Trautwein, McDonald & Pelzer, 1990; Carmeliet, 1992). Thus, despite the use of Na⁺-free pipette solution in the 2·2T experiments on perforated-patch myocytes, it was probable that the increase in outward current and emergence of inward tail currents (see Fig. 5) was, as in the myocytes of Fig. 7, due to development of a large Na⁺—Ca²⁺ exchange current ($I_{\text{NaCa}}$). To examine this possibility, we measured the effects of two $I_{\text{NaCa}}$-inhibiting procedures (addition of 5 mM Ni²⁺ (Kimura, Miyamae & Noma, 1987) and removal of external Na⁺) on membrane currents in perforated-patch myocytes treated with 2·2T solution. Outward $I_{\text{Na}}$ which was elevated after 8 min treatment with 2·2T solution quickly declined after each of the two $I_{\text{NaCa}}$-inhibiting interventions (Fig. 8A and C). On average, Ni²⁺ and Na⁺ removal after 8–13 min 2·2T treatment inhibited the $I_{\text{Na}}$ induced at +40 mV (0·8 ± 0·1 nA, n = 6) by 77 ± 3 and 65 ± 17 %, respectively (n = 3 each). These two interventions also inhibited the tail currents flowing on repolarization from positive potentials (Fig. 8B and D); tail currents induced at −100 mV (−0·6 ± 0·1 nA, 20 ms post-repolarization, n = 6) were inhibited by 88 ± 14 and 90 ± 7 %, respectively (n = 3 each).

**Action potentials under modified conditions**

The voltage-clamp results suggest that elevations of intracellular Na⁺, Ca²⁺ and K⁺ concentrations may underlie many of the action potential changes observed in impaled myocytes superfused with hyperosmotic solutions. To obtain additional information on this point, we investigated the effects of altering the stimulation rate, buffering intracellular Ca²⁺ and nulling the hyperpolarization, on action potentials in impaled myocytes subjected to strong hyperosmotic challenges.

**Figure 9. Effects of stimulation rate on action potential configuration in impaled myocytes superfused with hyperosmotic solution**

*Fig. 9A.* effect of 1 min rests during regular 1 Hz stimulation. The labels indicate the steady-state (SS) pre-test and the first post-rest (PR1) action potentials recorded before and during 2·8T treatment. The dashed lines indicate 0 and −100 mV levels. *Fig. 9B.* effect of 2·8T superfusion on action potential configuration in a myocyte stimulated at 2 Hz. The higher driving rate was imposed for 22 min, beginning 2 min prior to the switch to 2·8T solution. The 17, 1 Hz record was obtained just before the change in stimulation rate, and the 17, 2 Hz record was obtained 115 s later. The 2·8T records were obtained at the times indicated. The dashed lines indicate 0 and −100 mV levels. *Fig. 9C.* and *Fig. 9D.* changes in the action potential durations of myocytes driven at 1 Hz (n = 8) (data from Fig. 3) or 2 Hz (n = 6) during 20 min exposures to 2·8T solution; *P < 0·05 versus 1 Hz data.*
Altered stimulation rate. Na\(^+\) and Ca\(^{2+}\) influx were modulated by changing stimulation rate. To reduce influx, regular stimulation (1 Hz) was stopped for 1 min just before and after 9 and 20 min 2·8 T superfusion. The first post-rest action potential under 17 conditions had a higher plateau and a moderately longer duration than the steady-state event (Fig. 9A, left). The effect of the rest after 9 min 2·8 T was similar but more pronounced, whereas the major effect after 20 min was the reappearance of a mid-repolarization hump (Fig. 9A; also see Fig. 2C). Similar results were obtained in two other myocytes rested at these times.

The opposite effect was recorded when Na\(^+\) and Ca\(^{2+}\) influx was promoted by driving myocytes at 2 Hz throughout the 20 min 2·8 T treatment. Although the pattern of changes (early depression followed by lengthening and subsequent collapse) was the same as during 1 Hz stimulation, the lengthening phase was abbreviated and the subsequent collapse much more severe (Fig. 9B–D).

Buffered intracellular Ca\(^{2+}\). Myocytes were impaled with microelectrodes filled with 0·3 m EGTA solution to assess the influence of intracellular Ca\(^{2+}\) accumulation on modifications of electrical activity induced by hyperosmotic solutions. During 20 min EGTA-loading periods in 1 T solution, stimulated contractions were rapidly abolished (visual observations), the action potential plateau phase became more prominent and the APD\(_{90}\) lengthened by 30% to 197 ± 14 ms (Table 3). These are the changes expected from a significant diffusion of pipette EGTA into the myoplasm and consequent slowing of inactivation of I\(_{Ca,L}\) (see Trautwein, Taniguchi & Noma, 1982; McDonald, Pelzer, Trautwein & Pelzer, 1994). Exposure of EGTA-loaded myocytes to 2·2 T or 2·8 T solution caused rapid hyperpolarization, depression of the overshoot and abbreviation of the action potential duration. Compared with the changes observed in non-EGTA-loaded myocytes, there was (i) more moderate early shortening of the APD\(_{10}\) and APD\(_{90}\) (ii) a subsequent rapid shortening (i.e. no intermediate lengthening), and (iii) no sign of a slowing of terminal repolarization (Fig. 10; also see Table 3 for absolute measurements). These results suggest that an accumulation of intracellular Ca\(^{2+}\) is involved in the rebound lengthening and slowed terminal repolarization observed in non-loaded myocytes.

Nulled hyperpolarization. Hyperpolarization of cardiac muscle cells exposed to hyperosmotic solution has been attributed to cell dehydration and resultant concentrating of [K\(^+\)]\(_i\) (e.g. Fozzard & Lee, 1976). Similar events in the myocytes examined here may have contributed to action potential shortening as a consequence of larger K\(^+\) gradients. This possibility was evaluated by elevating extracellular [K\(^+\)] during 40 min treatments of EGTA-loaded myocytes with hyperosmotic solution. The concentration of K\(^+\) required to restore the transmembrane K\(^+\) gradient (and resting potential) to pretreatment value was calculated by using the Nernst equation and the ARP after 18 min treatment (see legend to Fig. 11). When K\(^+\) (2·8 ± 0·2 m\((m\) 2·2 T, 3·7 ± 0·2 m\((m\) 2·8 T; \(n = 4\) each) was added to hyperosmotic solution after 20 min treatment, hyperpolarization was effectively nulled and there was a progressive increase in the overshoot and duration of the action potential (Fig. 11; Table 3). These changes were fully reversed when the extra K\(^+\) was removed (Fig. 11B), suggesting that an augmented transmembrane K\(^+\) gradient directly or indirectly contributes to the action potential shortening caused by hyperosmotic solution.

DISCUSSION

The action potential in guinea-pig ventricular myocytes underwent a biphasic change (minor shortening, minor lengthening) during exposure to 1·2–1·5 T solution and a triphasic change (marked shortening, lengthening and secondary shortening) during exposure to 2·2–2·8 T

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Table 3. Action potential data measured from non-loaded and EGTA-loaded myocytes before and during prolonged superfusion with 2·2 T and 2·8 T hyperosmotic solutions

| Relative osmolality | [K\(^+\)]\(_i\) \((\text{mM})\) | Non-loaded myocytes | EGTA-loaded myocytes |
|---------------------|-----------------|------------------|---------------------|
|                     | [mV]            | [ms]             | [ms]               |
| 1 T                 | 4 · 6           | −90 ± 0·5 (15)   | 84 ± 6 (15)        | −90 ± 0·5 (8)       |
| 2·2 T               | 4 · 6           | −101 ± 1·0 (7)*  | 37 ± 7 (7)*        | −103 ± 0·4 (4)*    |
| 2·2 T               | 7·4 ± 0·2       | —                | —                  | −91 ± 0·4 (4)†     |
| 2·8 T               | 4 · 6           | −107 ± 0·7 (8)*  | 6 ± 1 (8)*         | −106 ± 1·3 (4)*    |
| 2·8 T               | 8·3 ± 0·2       | —                | —                  | −90 ± 0·9 (4)†     |

Myocytes stimulated at 1 Hz were equilibrated in 1 T solution for 20 min and then superfused with 2·2 T or 2·8 T Tyrode solution (4·6 m\((m\ [K\(^+\)]) for 20 min, EGTA-loaded myocytes were then exposed to 2·2 T or 2·8 T solution that contained elevated [K\(^+\)]\(_i\) for an additional 10 min. Values are means ± s.e.m.; number of myocytes indicated in parentheses. * \(P < 0·05\) versus pooled within-group 1 T values, † \(P < 0·05\) versus values from non-loaded myocytes.
solution. In an earlier study on guinea-pig ventricular papillary muscles, Hermosyler et al. (1972) reported that 2T solution for 20–30 min depressed the action potential overshoot and plateau, reduced the duration at 50% repolarization to 30% control and slowed terminal repolarization (i.e. steady-state action potential similar to the 20 min 2·2–2·8T events shown in Fig. 2). Ehara & Hasegawa (1983) later reported that 1·5T solution prolonged the APD_o in guinea-pig papillary muscle to 120% control prior to a shortening to control value at 30 min. They noted that 2T solution induced more pronounced lengthening and more pronounced subsequent shortening but provided no data. In summary, with the exception that early shortenings were not detected in papillary muscles, the present results are in broad agreement with those of the tissue studies. We begin by discussing the early shortening in the myocytes, and then consider rebound lengthening and secondary shortening before turning to the modified responses of EGTA-loaded myocytes.

**Early changes in electrical activity**

Hyperpolarization of the resting membrane, reduction of the overshoot amplitude, abbreviation of the plateau and a speeding up of subplateau repolarization were observed within 1–2 min of switching from 1T to a hyperosmotic superfusate. These changes were larger the more severe the hyperosmotic challenge, and they appear to have been primarily due to elevations in intracellular K⁺, Ca²⁺ and Na⁺ concentrations caused by rapid dehydration of the myocytes.

In an earlier study on rabbit ventricular muscles impaled with K⁺-selective microelectrodes, Fozzard & Lee (1976) found that superfusion with 1·5T sucrose solution caused a hyperpolarization that was attributed to a 33% increase in intracellular K⁺ activity. In the present study, additions of small amounts of external K⁺ that were calculated using the Nernst equation effectively nulled the hyperpolarizations induced by 2·2–2·8T solution. The average AEP of −12 ± 0·5 mV caused by 2·2T solution suggests that there was a 58 ± 3% increase in myocyte [K⁺]. Other factors being constant, this would have required a 37 ± 1% shrinkage in cell water (i.e. relative cell water = 1/relative [K⁺]) which, assuming an osmotically inactive cell volume of 18–34% (see Roos, 1986; Drewnowska & Baumgarten, 1991), translates to a 24–30% shrinkage in cell volume, in reasonable agreement with the 33 ± 1% shrinkage calculated from myocyte dimensions.

In addition to hyperpolarization, an increase in [K⁺] should increase K⁺ channel conductance. However, for reasons that are unclear, hyperosmotic solution actually inhibited the delayed-rectifier I_K (cf. Sasaki et al. 1994), a response that has a lengthening influence on the action potential. On the

**Figure 10. Effects of 2·2T and 2·8T solutions on the action potential configuration in myocytes impaled with microelectrodes filled with 0·3 m EGTA solution**

A and C, example records from two myocytes impaled with microelectrodes filled with 0·3 m EGTA solution (EGTA_pip). The dashed lines in each panel indicate 0 and −100 mV levels. B and D, comparison of the effects of hyperosmotic solution on action potential duration in EGTA-loaded myocytes (n = 4 each for 2·2T and 2·8T) and non-loaded myocytes (n = 7 and 8 for 2·2T and 2·8T, respectively; data from Fig. 3). Stimulation rate 1 Hz. Values are presented as means ± s.e.m.; * P < 0·05 versus non-loaded myocytes. See Table 3 for absolute values.
other hand, an [K⁺]-mediated increase in the inward rectifier K⁺ conductance should enhance sub-plateau time-independent current and repolarization rate, and both of these effects were in fact observed during hyperosmotic treatment.

Shrinkage-induced elevations of [Ca²⁺] and [Na⁺] appear to be responsible for two changes in membrane current that can explain early shortening of the action potential plateau. The first of these changes, an inhibition of \( I_{Ca,L} \), is a well-established consequence of elevated [Ca²⁺] in guinea-pig ventricular myocytes (see McDonald et al. 1994; You et al. 1994). The second change, a rapid increase in outward current, was most probably related to elevated [Na⁺], because the induced current was: (i) large in myocytes that were dialysed with 10 mM Na⁺ solution; (ii) absent in myocytes dialysed with Na⁺-free solution; and (iii) suppressed by removal of external Na⁺ (which should lower [Na⁺]). The shape of the current waveform at positive potentials in Na⁺-dialysed cells (e.g. Fig. 7C), and its inhibition by Ni²⁺, point to involvement of Na⁺–Ca²⁺ exchange. Since outward \( I_{NaCa} \) at positive potentials increases with [Na⁺], the degree of plateau suppression at early times is expected to increase with the degree of shrinkage-induced elevation of [Na⁺], (external osmolality) in a manner similar to that shown in Fig. 3.

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**Figure 11.** Action potentials in impaled EGTA-loaded myocytes after nulling of hyperosmotically induced hyperpolarization with external K⁺

Myocytes loaded with EGTA (EGTApip) were superfused for 20 min with 4.6 mM K⁺ 2.2T or 2.8T. The superfusate was then exchanged for a hyperosmotic solution that contained higher K⁺ (7.4 ± 0.2 mM for 2.2T solution and 8.3 ± 0.2 mM for 2.8T solution, n = 4 each) for 10 min. The concentration of K⁺ required to offset the hyperosmotically induced ΔRP was calculated as 4.6 x 10⁻³[ΔRP/K⁺] on the basis of the theoretical 61 mV Nernstian slope. *A*, example records obtained from a myocyte during 2.8T treatment. The K⁺ concentration of the 2.8T solution (4.6 mM K⁺, open box) was increased by 3.8 mM at 20 min (lower hatched box). The dashed lines in each panel indicate the 0 and −100 mV levels. *B*, time plots of ΔRP, APD₅₀, and APD₉₀ before, during and after elevation of external K⁺ (lower hatched box) from standard 4.6 mM (open box). Stimulation rate 1 Hz. Values are presented as means ± s.e.m. (n = 4 myocytes each for 2.2T and 2.8T solutions). See Table 3 for absolute values.
In addition to elevating [K\(^+\)], [Na\(^+\)], and [Ca\(^{2+}\)], hyperosmotic shrinkage will have affected the intracellular concentrations of other ions as well. Amongst the latter, the most likely to have exerted rapid effects on the action potential are H\(^+\) and Mg\(^{2+}\). An increase in intracellular H\(^+\) inhibits most cation-selective channels (Hille, 1992; McDonald et al. 1994), but any such inhibition in the myocytes was probably negligible. The reason is that the changes in cardiac intracellular pH induced by hyperosmotic treatment appear to be relatively minor (e.g. reduction of 0·1 unit by 2T solution: Whalley et al. 1984) compared with the large changes (> 1·0 unit) required for significant inhibition of channel current (e.g. I\(_{\text{Ca,L}}\), Irisawa & Sato, 1986; McDonald et al. 1994). A similar argument can be made against an important role for shrinkage-elevated intracellular Mg\(^{2+}\). For example, an increase in intracellular [Mg\(^{2+}\)] from 1·3 to 5·0 m\(\text{M}\) was required to block 50\% of I\(_{\text{Ca,L}}\) (Agus, Kelepouris, Dukes & Morad, 1989), and it is difficult to see how intracellular [Mg\(^{2+}\)] (normally around 1 m\(\text{M}\) in heart cells: see McDonald et al. 1994) could have risen to anywhere near this level in the myocytes studied here.

Rebound lengthening and secondary shortening of the action potential

The relatively small rebound lengthening of the action potential during moderate challenge was mainly due to a slowing of repolarization in the region between +20 and −20 mV (Fig. 2A). Since the rate of change of voltage in this potential region is normally quite slow, even small reductions in net outward current can lengthen the action potential. The voltage-clamp results point to two changes that reduced net outward current: (i) an inhibition of the outward-directed delayed rectifier I\(_{\text{K1}}\) and (ii) an increase in inward current due to a slowing of the inactivation of I\(_{\text{Ca,L}}\). The slowing of the inactivation of I\(_{\text{Ca,L}}\) during hyperosmotic treatment is quite unexpected in view of the acceleratory effect of elevated [Ca\(^{2+}\)]\(_{\text{cyt}}\) (McDonald et al. 1994). A similar argument will be moderated by rising osmolarity-related technical problem or obscure overlapping results do not warrant speculation on the cause of the effect of elevated [Ca\(^{2+}\)]\(_{\text{cyt}}\). However, the effect of [Ca\(^{2+}\)]\(_{\text{cyt}}\) on outward I\(_{\text{SACa}}\) will be moderated by rising [Ca\(^{2+}\)]\(_{\text{cyt}}\), which shifts the reversal potential of I\(_{\text{SACa}}\) to more positive potentials than would otherwise be the case and thereby reduces outward I\(_{\text{SACa}}\). Under moderate hyperosmotic conditions, the latter effect may help sustain the plateau.

Under severe hyperosmotic conditions, the reversal-shifting effect of rising [Ca\(^{2+}\)]\(_{\text{cyt}}\) with additional boosts caused by stimulated Ca\(^{2+}\) release (Allen & Smith, 1987), sets the conditions required for the two phases of subplateau lengthening. In regard to the first phase, large inward-directed I\(_{\text{SACa}}\) at ca. −30 mV may almost completely offset the relatively weak inward rectifier K\(^+\) current (I\(_{\text{K1}}\)) at this potential and create a hump in repolarization (Fig. 2C). Repolarization proceeds slowly as I\(_{\text{SACa}}\) decays and then more quickly with growing I\(_{\text{K1}}\) at potentials more negative than −40 mV. The second phase of lengthening, at potentials nearer the resting potential, is attributed to residual inward I\(_{\text{SACa}}\) that, enhanced at this voltage, can transiently offset a fraction of voltage-attenuated I\(_{\text{K1}}\) and thereby delay repolarization. The latter phase of repolarization became more and more prominent as secondary shortening gained momentum. The secondary shortening was led by a collapse of the plateau, consistent with progressive inhibition of I\(_{\text{Ca,L}}\) and growth of outward I\(_{\text{SACa}}\). The growth of outward I\(_{\text{SACa}}\) seems to be linked to a negative shift in the reversal potential of I\(_{\text{SACa}}\) as indicated by negative shifts in hump potentials and their eventual merging with accentuated terminal repolarization phases (Fig. 2C). This pattern can be explained by an increasing [Na\(^+\)] (combined, perhaps, with ineffecual Ca\(^{2+}\) release under the Ca\(^{2+}\)-overload condition: Lakatta, 1992). Consistent with this interpretation, a 1 min rest period encouraged the re-emergence of a midrepolarization hump (Fig. 3A, right).

The action potential in myocytes loaded with EGTA

The effects of hyperosmotic superfusion on action potential configuration in EGTA-loaded myocytes were investigated to provide direction on the role of elevated [Ca\(^{2+}\)]\(_{\text{cyt}}\). An important finding was that rebound lengthening did not occur in EGTA-loaded myocytes; the action potential quickly shortened at all repolarization levels on switching from 1T to 2·2–2·8T solution, and remained that way during 20 min observation periods. The shortening occurred despite the strong likelihood that hyperosmotically induced inhibition of I\(_{\text{Ca,L}}\) was less severe in these myocytes than in non-loaded ones. A plausible explanation for the shortening is that in the absence of the moderating effect of high [Ca\(^{2+}\)]\(_{\text{cyt}}\) on outward I\(_{\text{SACa}}\) (see above), high [Na\(^+\)] generated outward I\(_{\text{SACa}}\) at all potentials above a projected I\(_{\text{SACa}}\) reversal potential near the resting potential.

When external [K\(^+\)] was increased to null the effect of hyperosmotically induced hyperpolarization in EGTA-loaded myocytes, there was a partial restoration of the overshoot and a significant prolongation of the plateau phase of the action potential. The elevation of external [K\(^+\)] may have affected plateau K\(^+\) currents by: (i) moderately
reducing outward driving force at positive potentials; and (ii) affecting K⁺ permeability with resultant overall moderate increase (Noble, 1979) or decrease (McDonald & Trautwein, 1978) in plateau K⁺ current. However, effects on K⁺ currents are unlikely to have been the major factor because restoration of the action potential configuration lagged behind the nulling of hyperpolarization (Fig. 11). A more suitable explanation is that action potential restoration reflected a time-dependent decline in [Na⁺], (and outward $I_{Na}$K) due to reduced Na⁺ influx during diastole and enhanced Na⁺ pumping. The reduced Na⁺ influx is a consequence of the positive shift in diastolic potential. For example, Eisner, Lederer & Vaughan-Jones (1981) have shown that [Na⁺] in glycoside-treated sheep Purkinje fibres declines with a time constant of ~3 min due to reduced inward leak when the membrane potential is shifted from a very negative potential to a more positive one. The enhanced Na⁺ pumping would be due to: (i) a shift in diastolic potential to a more favourable positive one in terms of voltage-dependent Na⁺ pumping (the dependence of cardiac Na⁺ pumping on voltage is steep at the potentials in question: Gadsby & Nakao, 1989); and, possibly (ii) a stronger direct activation of the external pump site if the $K_{Na}$ for K⁺ activation is as high as 2.6 mM in guinea-pig ventricular myocytes (see Gao, Mathias, Cohen & Baldo, 1990).

Concluding remarks

The consequences of hyperosmotic stress on intracellular ion concentrations, membrane ionic currents and cell electrical activity portend complex effects on the contractile performance of working myocardium. Elevation of [Na⁺], and [Ca²⁺], in concert with prolongation of the action potential (moderate hyperosmotic conditions) will enhance contractility, whereas larger increases have a negative inotropic effect (e.g. Allen & Smith, 1987).

To obtain a coherent view of the effects of hyperosmotic solution on cardiac electrical activity, it was necessary to investigate an extended range of hyperosmotic conditions (1·2—2·8 mosmol (kg H₂O)⁻¹) that clearly falls outside the bounds of interstitial osmolality normally experienced by the heart. However, it is worth noting that intravenous mannitol, which raises serum osmolality up to 420 mosmol (kg H₂O)⁻¹, has been used in the treatment of acute renal failure, acute brain oedema and acute glaucoma (e.g. Borges, Hocks & Kjellstrand, 1982), that patients in diabetic hyperosmolar coma may have serum osmolality up to 540 mosmol (kg H₂O)⁻¹ (~1·8 T) (Son, Rao, Bajaj & Treaser, 1990) and radiocontrast medium injected in the coronary system is strongly hypertonic (500—1600 mosmol (kg H₂O)⁻¹; Lieberman, 1992).
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Author's email address

T. F. McDonald: tenonce.mcdonald@dal.ca

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