Ionic basis for membrane potential changes induced by hypoosmotic stress in guinea-pig ventricular myocytes

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Abstract

Objective: Causal relation between changes in action potentials and activation of several ionic currents during hypoosmotic challenge was investigated. Methods: We recorded changes in membrane potentials and currents during hypotonic stress in guinea-pig ventricular myocytes using whole-cell patch-clamp technique. Results: Exposure of ventricular myocytes to hypotonic solution (0.6 T) caused initial prolongation (−107% of control) of action potential duration at 90% repolarization (APD90) in 65% of examined myocytes. Later shortening (−75% of control) of APD90 and depolarization of resting potential (RP) (−4 mV) developed in all cells. Initial prolongation of APD90 in hypotonic solution was mainly caused by transient activation of Gd+-sensitive non-selective cation (NSC) current. Late changes after ~180 s in hypotonic solution were sustained increase in slow component of delayed rectifier K+ current (Iks) in all cells, and activation of IClswell in 40% of cells. Prevention of APD90 shortening by chromanol, a selective blocker of Iks, was seen in about 40% of myocytes due to short APD in our experimental conditions. Application of 1 mM anthracene-9-carboxylic acid (9-AC) partially inhibited APD shortening in three of seven cells. Depolarization of RP was unaffected by the above-mentioned drugs, but was dependent on [K+]o. Conclusions: Initial prolongation followed by later shortening of APD in hypotonic solution are mostly caused by different sequences of NSC, Iks and IClswell currents activation. Depolarization of RP in hypotonic solution is probably due to dilution of subsarcolemmal K+ concentration and/or change in permeability ratio for Na+ and K+.

Keywords: Cl-channel; Ion channels; K-channel; Arrhythmia (mechanisms); Membrane potential; Repolarization; Stretch/m–e coupling

1. Introduction

Hypotonic stress is associated with serious disturbances of cardiac functions [1–3]. Significant changes in membrane potentials and currents in cardiac cells during exposure to hypotonic solution have been reported. Initial prolongation and secondary shortening of action potential duration (APD), accompanied by gradual depolarization of resting potential (RP) have been documented [4–7]. There have also been demonstrated activation of non-selective cation channel (NSC) [8,9], swelling-activated Cl−-channel (IClswell) [10–12], Na+ /K+ pump current [13] and stimulation of the slow component of delayed outward K+ current (Iks) [14,15]. The ionic basis for changes in APD and RP during hypotonic stress are not well clarified in terms of different current activation. For instance, Vandenberg et al. reported 4,4'-diisothiocyanato-stilbene-2,2'-disulfonic acid (DIDS), a blocker of IClswell partly blocked secondary APD shortening in about 20% of cells, and slightly reversed depolarization of RP [7]. On the other hand, Du and Sorota have shown that depolarization of RP in canine ventricular myocytes developed without activation of IClswell, opposite to the findings in canine atrial cells [5]. Thus, improved understanding of these mechanisms requires further investigation of the relation between time-course of current activation and membrane potential changes during hypotonic stress. Using pharmacological tools and isolation of specific currents, we examined the...
mechanisms of initial prolongation and secondary shortening of APD as well as depolarization of RP.

2. Methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996), and was in accordance with the institutional guidelines for animal care at Tokyo Medical and Dental University.

2.1. Myocytes preparation

Isolation of single ventricular myocytes from guinea-pig heart was carried out by an enzymatic dissociation procedure described previously [16]. Briefly, guinea-pigs, weighing 300–400 g were anesthetized with pentobarbital sodium (20–30 mg/kg, i.p.). After dissection from thorax under artificial respiration, hearts were perfused retrogradely with use of Langendorff apparatus. First, normal Tyrode solution was used for 5–10 min, and then nominally Ca\(^{2+}\)-free solution for 4 min, followed by perfusion with Ca\(^{2+}\)-free solution + collagenase (60 mg%, type II; Worthington Biochemical, Lakewood, NJ, USA) for about 20 min. The temperature of the solutions was kept at 35–37°C. Isolated cells were stored at room temperature in high-K\(^+\), low-Cl\(^-\) solution and used within 9 h from isolation. Cells were transferred to a bath on the stage of an inverted microscope (Diaphot TMD, Nikon, Tokyo, Japan) and were perfused with isotonic or hypotonic solution at 36 ± 0.5°C. The average speed of perfusion was about 1.5 ml/min by gravity and exchange of the bath solution was completed within 20 s.

Table 1
Composition of the solutions (in mM)

| Components     | External solutions | Standard | NSC | I\(_{\text{Clswell}}\) | Pipette solutions | Standard | NSC | I\(_{\text{Clswell}}\) |
|----------------|--------------------|---------|-----|-----------------------|-------------------|---------|-----|-----------------------|
| 1. NaCl        |                    | 64      | –   | –                     | –                 | –       | –   | –                     |
| 2. KCl         |                    | 4.0     |    | –                     | –                 | –       | –   | –                     |
| 3. CaCl\(_2\)  |                    | 1.8     |    | 2.0                   | 0.02              | –       | –   | –                     |
| 4. MgCl\(_2\)  |                    | 0.53    | 2.53| 0.53                 | –                  | 2.0     | 2.0 | –                     |
| 5. Glucose     |                    | 5.5     | 10  | 10                    | –                  | –       | –   | –                     |
| 6. HEPES       |                    | 5.0     | 10  | 10                    | 5.0               | 10      | 10  | 10                    |
| 7. K\(^+\)-aspartate |                | –       | –   | –                     | 100               | –       | –   | –                     |
| 8. Mg\(^{2+}\)-ATP |                | –       | –   | –                     | 5.0               | 5.0     | 5.0 | 5.0                   |
| 9. K\(^+\)-CP   |                    | –       | –   | –                     | 5.0               | –       | –   | –                     |
| 10. EGTA       |                    | –       | –   | –                     | 0.05              | 10      | 10  | 10                    |
| 11. CsCl       |                    | –       | 44  | –                     | –                 | 130     | 130 | 130                   |
| 12. TEA-Cl     |                    | –       | 20  | 60                    | –                 | 20      | 20  | 20                    |
| 13. Mannitol   |                    | 150     | 150 | 150                  | –                 | –       | –   | –                     |

NSC, for non-selective cation channel isolation; I\(_{\text{Clswell}}\) for isolation of I\(_{\text{Clswell}}\); CP, creatine phosphate; TEA, tetraethylammonium.

2.2. Solutions

The composition of the solutions used in this study are presented in Table 1. Standard isotonic external solution was obtained from normal Tyrode solution by reducing NaCl concentration to 64 mM and addition of 150 mM mannitol (osmolality was 282 ± 15 mOsm/l, measured by Vapor Pressure Osmometer 5520 (Wescor, Utah, USA). Osmolality of the pipette solution was 270 ± 11 mOsm/l. The standard hypotonic solution was the same as the isotonic except mannitol was reduced to 50 mM (osmolality was 177 ± 11 mOsm/l). pH was adjusted to 7.3 in the bath solution with NaOH, and 7.25 in the pipette solution with KOH. Some experiments were performed in the presence of 0.2 mM Cd\(^{2+}\) or 1 μM nisoldipine to block L-type Ca\(^{2+}\) current (I\(_{\text{Ca,L}}\)) and 0.1 mM ouabain to block Na\(^+\)/K\(^+\) pump current. The bath solution used for isolation of NSC had pH = 7.3 adjusted with TEA-OH and the pipette solution to pH = 7.25 with CsOH. The external hypotonic solution for NSC recording was the same except mannitol concentration of 50 mM. In the bath solution used for isolation of I\(_{\text{Clswell}}\), pH was 7.3 with TEA-OH and in the pipette solution pH = 7.25 with CsOH. The external hypotonic solution was similar, except reduced mannitol concentration to 50 mM. For isolation of I\(_{\text{Ca,L}}\), we used similar bath and pipette solutions as for isolation of I\(_{\text{Clswell}}\) (Table 1).

2.3. Drugs

Gadolinium(III) chloride (Aldrich, Milwaukee, WI, USA) was dissolved in distilled water, freshly prepared before each experiment and added at final concentration of 20 μM to the test solution. Addition of this concentration of Gd\(^{3+}\) for up to 10 min to cells perfused with isotonic solution had no significant effect on APD (148 ± 4 ms in the absence, and 149 ± 4 ms in the presence, n = 8) and RP.
(−78.5±0.9 mV in the absence and −79.3±0.3 mV in the presence, not significant). Anthracene-9-carboxylic acid (9-AC) and DIDS (Sigma Chemical, St Louis, MO, USA) were dissolved in dimethylsulfoxide (DMSO) to give a stock solution of 100 mM, freshly prepared, given in a final concentration of 1 mM (9-AC) or 0.5 and 2 mM (DIDS) in light-proof container. E-4031 (a gift from Eisai Pharmaceutical Comp., Tokyo, Japan) was dissolved in distilled water, freshly prepared before each experiment and used at a concentration of 5 μM. Chromanol (a gift from Prof. H. J. Lang, Hoechst AG, Frankfurt, Germany) was dissolved in DMSO given a stock solution of 10 mM, while final concentration in the bath was 10 μM to 30 μM. Cadmium-chloride was dissolved in distilled water. Nisoldipine was prepared as a 1 mM stock solution in DMSO. Ouabain was dissolved in DMSO, to give the stock solution of 50 mM, using a final concentration of 0.1 mM. DMSO was added at the same concentration to all isotonic and hypotonic solutions when drugs dissolved in this solvent were used.

2.4. Electrophysiology

Action potentials and membrane currents were recorded by a whole-cell patch-clamp technique using a patch-clamp amplifier (Axopatch 200B, Axon Instrument, Foster City, CA, USA). Suction pipettes were made from borosilicate glass capillaries with inner filaments (Clark Electromedical Instruments, Pangbourne, UK) pulled with a micro-electrode puller (PP-83, Narishige, Tokyo, Japan). The tip resistance of typical electrode when filled with standard internal solution was 1.5–2.5 MΩ. A 2 M KCl agar bridge between bath solution and AgCl-reference electrode immersed in pipette solution was used to minimize liquid junction potential. The membrane potential and current signals were filtered at 2 kHz, monitored by a storage oscilloscope DCS 7040 (Kenwood, Tokyo, Japan), digitized using an analog converter (Digidata 1200, Axon Instruments, Foster City, CA, USA) and stored in a personal computer (Fujitsu, Tokyo, Japan) for later analysis. Action potential was elicited by injecting supra-threshold currents of 3 ms duration through the recording electrode at a frequency of 0.1 Hz. Various ramp and voltage step protocols were used as indicated (see Results) for current measurement. Membrane capacitance was calculated from the integral of the current transient in response to 10-mV pulses. pCLAMP software (Version 6.0.4, Axon Instruments) was used to generate pulse protocols and for data acquisition. Origin software (Version 5.0) was used for data analysis.

2.5. Experimental protocols

All measurements of membrane potentials and currents were performed at least 7 min after obtaining a whole-cell configuration. Additional 3 min were allowed to watch stability of action potential configurations and/or membrane currents before collecting the control data. As initial prolongation of APD did not develop in all examined myocytes, we arranged a pilot study to examine whether cells with initial prolongation during first exposure to hypotonic solution showed similar prolongation on the second exposure to hypotonic solution. In six out of six preparations with initial prolongation of APD during the first short exposure to hypotonic solution (150 s, enough time for initial prolongation to appear and subside), the second exposure to hypotonic solution induced a similar phenomenon. Thus, only the cells with initial prolongation during first exposure to hypotonic solution were examined with Gd³⁺, Cd²⁺ or E-4031.

2.6. Statistical analysis

Values are means±S.E.M. Two-way ANOVA with Newman–Keul’s test, Student’s t-test for paired samples and non-parametric Kruskal–Wallis test were used for statistical analysis as it was appropriate. P<0.05 was considered significant.

3. Results

3.1. Effects of hypotonic perfusion on membrane potentials

Using current-clamp mode, we monitored the time-course of changes in action potential duration at 90% repolarization (APD₉₀) and resting potential (RP). Exposure to hypotonic solution produced an initial prolongation of APD₉₀ (within 60 s of exposure) in 21 out of 33 examined cells, followed by later shortening of APD₉₀ in all preparations. In the remaining 12 myocytes, shortening without initial prolongation of APD₉₀ appeared. Depolarization of RP started almost simultaneously with shortening of APD₉₀, after about 120 s perfusion with hypotonic solution in all examined preparations (Fig. 1A and B). APD₉₀ after 60 s in hypotonic solution was prolonged from 180±8 ms in the control isotonic solution to 192±9 ms (n=21, P<0.05) and the rest of myocytes slightly shortened APD₉₀ from 170.5±7 ms to 163.8±8 ms (n=12, NS). Then, APD₉₀ gradually shortened to 132±5 ms (n=33, P<0.01, in comparison with control isotonic solution) at 300 s in the hypotonic solution. At the same time, a small and gradual depolarization of RP developed and the average was −73.9±0.4 mV after 300 s, compared to −78.2±0.7 mV in the control (n=33, P<0.05).

3.2. Effects of hypotonic solution on whole-cell membrane currents

Using the same external and pipette solutions as for recording the membrane potential changes, we recorded
Fig. 1. Effects of hypotonic solution on membrane potentials of guinea-pig ventricular myocytes. (A) Time-course of changes in APD_{90}; ○, average data for 21 cells with initial prolongation of APD_{90}; □, average data for 12 cells without initial prolongation of APD_{90}. (B) Time-course of changes in resting potential (RP); ◼, average data for cells with initial prolongation of APD_{90}; □, average data for cells without initial prolongation of APD_{90}. Values are means±S.E.M. from six experiments. (C) Superimposed original traces of action potential recorded from a representative myocyte at the time indicated by the same symbols as in A and B. (a) Control; (b) after 60 s exposure to hypotonic solution; (c) after 300 s exposure to hypotonic solution; (d) washout. *P<0.05, **P<0.01; significant differences from the control in isotonic solution.

whole-cell currents elicited by the fast descending ramp protocol (46 mV/s) in hypotonic solution (Fig. 2). During early period (less then 100 s) of hypotonic stress, a small but significant decrease in outward currents at positive voltages was observed (b). At other voltage regions no remarkable current changes were noted. After 150 s and later in hypotonic solution, gradual increase in outward current at positive voltages developed to exceed the control level. At the same time, outward currents at ~80 mV, roughly corresponding to the RP level, were slightly but significantly suppressed. Inward currents at voltages negative to ~80 mV were increased (c) with a positive shift of the reversal potential (4.4±0.9 mV from the control, n=5). However, the slope of negative current portions was not changed (54.8±4.9 nS in isotonic solution vs. 51.1±2.5 nS in hypotonic solution, n=5, NS).

3.3. Early changes in membrane currents during exposure to hypotonic solution

We assumed that decrease in outward current (I_{Kt} and I_{Kr}) or increase in inward current (pump current, I_{Ca,L}, NSC) could underlie observed change in whole-cell current during the first 100 s in hypotonic solution. The increased pump current was not likely a candidate, since we used pipette solution without Na^+ and the presence of Na^+ was mandatory to activate this current [14]. Moreover, addition of 100 μM ouabain did not affect transient depression of whole-cell current mentioned above (n=4, data not shown). Therefore, we recorded changes in isolated I_{Kt}. Fig. 3 shows changes in tail currents in hypotonic solution in the presence of 5 μM E-4031 to block I_{Kr} and 1 μM nisoldipine to block I_{Ca,L}. Normalized data obtained from five cells (Fig. 3D) clearly indicated that initial (between 30 and 100 s) suppression of tail current (from 0.74±0.24 pA/pF in the control to 0.58±0.19 pA/pF; n=5, P<0.05) was followed by later enhancement to 1.5±0.36 pA/pF after 300 s in hypotonic solution (P<0.01 vs. control). Chromanol at 30 μM completely inhibited increase in the tail current.

To rule out participation of I_{Kt} and I_{Ca,L} in initial prolongation of APD_{90}, we examined time-course of changes in isolated I_{Kt} and I_{Ca,L} during hypotonic challenge (Fig. 4). As to changes in I_{Kt}, the current was stable...
Fig. 2. Effects of hypotonic challenge on the whole-cell current elicited by fast ramp protocol. Voltage protocol is shown in Inset. Bath and pipette solutions are the same as in Fig. 1. (A) Average data for time-course of normalized whole-cell current in hypotonic solution at +30 mV and −80 mV. (B) Original traces from a typical myocyte: (a) control; (b) after 60 s exposure to hypotonic solution; (c) after 300 s exposure to hypotonic solution; (d) washout. (symbols have the same meaning as in the Fig. 1.) *P<0.05, **P<0.01; significant differences from the control in isotonic solution.

Fig. 3. Time-course of $I_{\text{Ktail}}$ current in hypotonic solution. Voltage protocol is indicated in the Inset in panel B (repolarization test pulse from +40 mV to −40 mV, every 20 s). The tail current upon repolarization (dash circle in Inset) is shown in A–C. Original traces recorded from one representative cell: (A) in isotonic solution; (B) after 60 s in hypotonic solution; (C) after 300 s in hypotonic solution; (D) average data from five cells; *P<0.05, **P<0.01; significant differences regarding control values in isotonic solution.
Fig. 5. Original superimposed current traces obtained in Na\(^+\), K\(^+\) and Ca\(^{2+}\)-free bath solution after 60 s of exposure to hypotonic solution in the absence and presence of Gd\(^3+\). (a) Control in isotonic solution, (b) after 60 s in hypotonic solution, (c) washout, (d) after 60 s exposure to hypotonic solution in the presence of Gd\(^3+\). (Inset: ramp-protocol used in this experiment).

not likely to participate in the initial prolongation of APD during hypotonic stress.

Literature data indicate that hypotonic challenge can induce early transient current sensitive to Gd\(^3+\), a blocker of NSC, in cardiomyocytes [17]. A small change in this current could be masked by overlaps with other currents in the presence of all standard ionic concentrations. Therefore, we used Na\(^-\), K\(^-\) and Ca\(^{2+}\)-free external solution and replaced Cs for K in the pipette solution to isolate NSC (see Methods for details). In this case, the only charge carriers should be Cs\(^+\) and Cl\(^-\). According to Nernst equation, reversal potential for Cs\(^+\) (\(E_{\text{Cs}}\)) was \(-28\) mV, and for Cl\(^-\) (\(E_{\text{Cl}}\)) was \(+23\) mV in our experimental condition. Under such condition we observed a current with almost linear I–V relationship and slight outward rectification in isotonic solution. Reversal potential of this current was \(-18\pm4\) mV (\(n=10\)). Application of 20 \(\mu\)M Gd\(^{3+}\) significantly reduced this current from \(0.43\pm0.1\) pA/pF to \(0.22\pm0.1\) pA/pF (at \(-60\) mV) and from \(-1.3\pm0.1\) pA/pF to \(-0.7\pm0.1\) pA/pF (at \(-100\) mV) (\(n=6, P<0.01\)). Therefore, it is conceivable to assume that Gd\(^{3+}\)-sensitive current in our experimental condition is NSC. In the same external and internal solutions, we could observe the activation of similar current as NSC in seven out of 10 myocytes after about 60 s in hypotonic solution only in the absence of 20 \(\mu\)M Gd\(^{3+}\) (Fig. 5). The activation of this current was transient and the current subsided after about 120 s in hypotonic solution.

3.4. Effects of Gd\(^{3+}\) and chromanol on initial prolongation of APD

The results in the previous section suggest that increased NSC or decreased \(I_{Kf}\) might participate to initial prolongation of APD\(_{90}\) in hypotonic solution. We tested effects of
Gd$^{3+}$ on action potential changes in hypotonic solution. In six out of seven examined myocytes with initial prolongation of APD$_{90}$, application of 20 μM Gd$^{3+}$ prevented the prolongation in the second exposure to hypotonic solution (Fig. 6). Gd$^{3+}$, however, did not affect depolarization of RP (4.03±0.6 mV in the absence vs. 3.75±0.3 mV in the presence, \( n=7 \), NS) and secondary shortening of APD$_{90}$ (27±3% in the absence vs. 23.5±5.5% in the presence, \( n=7 \), NS). As Gd$^{3+}$ is not a pure blocker of NSC and can affect \( I_{ca,l} \) and \( I_{kr} \) as well [18,19], abolition of APD changes by Gd$^{3+}$ may not be attributed to involvement of NSC, but the results described in the previous section exclude possible contribution of \( I_{ca,l} \) and \( I_{kr} \).

Although our current measurements suggested a decrease in \( I_{kr} \) during early phase of hypotonic stress, contribution of this current to determine APD in our experimental conditions seemed to be negligible. The average APD$_{90}$ of myocytes in isotonic external solution containing 64 mM NaCl was 176±7.5 ms (\( n=33 \)). As the duration of APD was critical for participation of \( I_{kr} \) in repolarization [20], we used action potential-clamp method to examine chromanol effects. When we used action potential-clamp with 200 ms or less APD$_{90}$, there was no chromanol-sensitive current elicited in isotonic solution and after hypotonic exposure for 100 s (data not shown). The chromanol-sensitive current was activated only after about 150 s in hypotonic solution with the 200 ms APD$_{90}$. Therefore, it is unlikely that transient depression of \( I_{kr} \) can be a major factor for initial prolongation of APD under our experimental conditions.

### 3.5. Late changes in membrane currents during exposure to hypotonic solution

According to the previous data, exposure of cardiomyocytes to hypotonic solution could increase time-dependent \( I_{kr} \) and time-independent currents \( I_{clswell} \) [10,21]. In the presence of Cd$^{2+}$ and ouabain, we observed hypotonic-sensitive time-dependent current in five out of five cells using voltage-step protocol shown in Inset in Fig. 7. The current increase was quickly and reversibly inhibited by 10 μM chromanol (Fig. 7C). Maximal increase in time-dependent current at +40 mV was 10.7±1.7 pA/pF after 300 s in hypotonic solution, from 4.3±0.9 pA/pF in the control (\( n=5 \), \( P<0.05 \)). Addition of chromanol reduced this current to 3.8±1.3 pA/pF (\( n=5 \), \( P<0.05 \) vs. in the absence of chromanol).

In order to examine time-independent current changes in hypotonic solution, we used fast descending ramp protocol in Na$^+$-, K$^+$- and Ca$^{2+}$-free solution with Cs$^+$ instead of K$^+$ in pipette solution (see Methods for the solutions) for 300 s exposure. In nine examined myocytes, hypotonic-sensitive current elicited by the ramp protocol was not affected significantly by DIDS, neither at 0.5 nor at 2.0 mM. We next used 9-AC, another blocker of \( I_{clswell} \) to examine hypotonic-sensitive Cl$^-$ current. Hypotonic-sensitive current was observed in four out of 10 cells, with a reversal potential of +19.6±1.6 mV, while predicted \( E_{clswell} \) was +21 mV. It was completely inhibited by 1 mM 9-AC after 2–3 min incubation (Fig. 7D). The remaining five myocytes were not elicited hypotonic-sensitive time-independent currents.

### 3.6. Effects of chromanol, DIDS and 9-AC on late shortening and depolarization of membrane potential in hypotonic solution

Application of 10 μM chromanol completely prevented the APD shortening in hypotonic solution in five of 13 myocytes (Fig. 8A), but did not affect it in eight preparations. Chromanol did not cause any significant change in RP depolarization during hypotonic perfusion. (4.2±0.2

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Fig. 6. Effects of 20 μM Gd$^{3+}$ on hypotonic solution-induced changes in membrane potential. (A) Typical example of action potential changes induced by hypotonic solution in the absence (A) and in the presence (B) of Gd$^{3+}$. A and B were recorded from a single myocyte. (a) Control in isotonic solution, (b) after 60 s in hypotonic solution, (c) after 300 s in hypotonic solution. (C, D) Summary of action potential changes from six out of seven examined myocytes with initial prolongation of APD$_{90}$ means that values in isotonic solution in each group were taken as 100%; the APD shortening in hypotonic solution in five...
mV in the absence vs. 4.25±0.3 mV in the presence, n=13, NS).

DIDS at 0.5 mM given for 30–50 s in hypotonic solution reversed partly and transiently the APD shortening in only three from 14 cells and had no significant influence on depolarization of RP (3.95±0.2 mV in the absence vs. 3.73±0.2 mV in the presence, n=6, NS). Increasing concentration to 2.0 mM did not improve efficacy of DIDS regarding APD shortening, but attenuated RP depolarization in some extent (3.9±0.2 mV in the absence vs. 2.8±0.2 in the presence, n=6, P<0.01). Prolonged incubation with DIDS for 2–3 min resulted in no remarkable effects (n=5, data not shown). Application of 9-AC prevented APD shortening by 49±11% of the control in three of seven myocytes (Fig. 8B), but no effects were noted in the other four preparations. Depolarization of RP was not affected by 9-AC (5.3±0.9 mV in the absence vs. 5.8±1.4 mV in the presence of 9-AC, n=5, NS).

3.7. Effects of different [K+]o on RP depolarization in hypotonic solution

Since neither above pharmacological treatments nor combination of them affected depolarization of RP in hypotonic solution, we examined changes in the background currents in bath solutions with different K+ concentrations. We used three different K+ concentrations (2, 4 and 20 mM) in external solution, keeping the osmotic difference constant between isotonic and hypotonic solutions as in previous experiments (Fig. 9A–C). Using 2 s voltage steps between −110 mV and −20 mV from holding potential of −40 mV, we observed a parallel rightward shift in reversal potential of background I–V curves in hypotonic solution, which increased with higher [K+]o. The shifts were for 2.3±0.6 mV at 2 mM, 4.4±0.9 mV at 4 mM and 5.5±0.8 mV at 20 mM [K+]o. Also, RP depolarization increased with increasing in [K+]o from 2.1±0.5 mV at 2 mM to 3.8±0.4 mV at 4 mM and 6.6±0.7 mV at 20 mM [K+]o (Fig. 9D).

4. Discussion

The major findings in this study are as follows: First, initial prolongation of APD90 observed in the majority of cells after about 60 s in hypotonic solution is mainly due to activation of NSC channels. Second, shortening of APD90 after about 180 s or later during hypotonic challenge is

![Fig. 7. Effects of chromanol at 10 μM (A–C) and 9-AC at 1 mM (D) on late time-dependent and time-independent current changes in hypotonic solution. (A–C) Changes in whole-cell currents (presented traces are obtained at +20 mV, +30 mV and +40 mV) elicited by voltage-step protocol in Inset. (D) Effects of 9-AC on isolated I_{Ca,vol}: superimposed traces of currents (elicited by the same ramp protocol as in Figs. 2 and 4). (a) Isotonic solution, (b) hypotonic solution, (c) hypotonic solution + 9-AC.](image)
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... demonstrated no significant change in $I_{\text{Ca,L}}$ during hypotonic stress together with the lack of effects of Cd$^{2+}$ on APD$_{90}$ prolongation. $I_{Kt}$ was depressed during hypotonic perfusion, which was in accordance with previous data [14,15], but the involvement of this current on early changes in APD was also excluded. We next explored possible contribution of $I_{Ks}$ and NSC to this phenomenon. Actually, time-dependent outward current at positive voltages compatible with $I_{Ks}$ was initially depressed during hypotonic stress, but chromanol, rather specific blocker of this current, did not prevent initial prolongation of APD$_{90}$. This discrepancy could be explained as too short APD$_{90}$ to significantly contribute to repolarization by $I_{Ks}$ in our experimental condition as revealed by the action potential-clamp experiments. Therefore, decreased $I_{Ks}$ as a factor for the initial prolongation of APD$_{90}$ seems negligible but a small contribution by this current may not completely be excluded.

Published data indicate that the NSC channel can be activated by hypotonic stress [8,9]. We could isolate NSC currents in our control solution and the currents were transiently activated in hypotonic solution. Both currents, as well as initial prolongation of APD, were easily blocked by 20 mM Gd$^3+$. While non-selective actions of Gd$^3+$ on $I_{Ks}$ and $I_{Clswell}$ [18,19] may suggest the participation of the two currents in these phenomenon, this possibility can be excluded by the reasons described above. Therefore, we assume that activation of NSC seems to be a main mechanism of initial prolongation of APD in hypotonic solution. The transient nature of this current activation, however, has not been explained in this study and its mechanism is to be further explored.

4.2. Secondary shortening of APD

There are several mechanisms proposed to be involved in this change of membrane potential. Activation of $I_{Clswell}$ [2,3,7] or ATP-sensitive K$^+$ current ($I_{\text{KATP}}$) [4,6], stimulation of $I_{Ks}$ [14,21] and dilution of intracellular ions [22] are the most plausible explanations. According to previous data activation of $I_{\text{KATP}}$ in hypotonic solution in the presence of 5 mM Mg-ATP, as we have in our pipette solution, can probably be excluded [4,6]. While specific conditions such as the presence of PIP$_2$ [23,24] have been shown to activate $I_{\text{KATP}}$ with physiological concentrations of cytoplasmic ATP, increase in PIP$_2$ concentrations under hypotonic stress is not documented. Moreover, we did not observe time-independent hypotonic-sensitive currents with reversal potential close to the reversal potential for K$^+$ when we applied fast ramp protocol (see Fig. 2). Enhancement of $I_{Ks}$ during hypotonic challenge was observed in our experiments in accordance with previous data [14,21]. A transient suppression of $I_{Ks}$ in hypotonic solution was not noted by Sasaki et al. [14]. This difference may be attributed to different experimental con-
Fig. 9. Influence of different [K⁺]₀ on changes in resting potentials and reversal potentials of background I–V curves induced by exposure to hypotonic solution. (A–C) Changes induced by hypotonic solution in background I–V relations in different [K⁺]₀. ○, Isotonic solution; ●, hypotonic solution. (D) Plot of changes in resting potentials (∆) and reversal potentials (□) against different bath K concentrations ([K⁺]₀). *P<0.05, **P<0.01; statistical differences from values at 2 mM of [K⁺]₀. • P<0.05; statistical difference from value at 4 mM of [K⁺]₀.

ditions, since they used K⁺-free bath solution. Although time-dependent current induced during hypotonic challenge was strongly suppressed by highly selective I𝐾 blocker chromanol [20,25], the drug effects on action potential shortening were not so remarkable. Chromanol prevented APD shortening in only 38% of myocytes (Fig. 8A). A lack of effect of chromanol in the rest (62%) of cells could be explained by too short APD to activate I𝐾 under our experimental condition (APD₀₀ about 170 ms) due to replacement of NaCl with mannitol. Therefore, it is possible that I𝐾 was not activated enough to participate in repolarization in all preparations in contrast to the condition under voltage-clamp depolarization where long (2 s) pulse was used. Probably, contribution of I𝐾 to APD determination depends on the basal length of APD in individual cells and amplitudes of other currents forming action potential repolarization.

Activation of I_Clswell [7] has been shown to contribute to action potential changes in hypotonic solution. We failed to inhibit time-independent currents induced by hypotonic solution by 0.5 mM DIDS and obtained inhibitory effects by the same concentration on APD shortening in 21% of the preparations. While the latter values on APD shortening were similar to previous data [7], our observed effects were transient and APD returned to pretreatment values in the continued presence of DIDS. Somewhat different responses between ours and results by Vandenberg et al. [7] appeared not to be due to experimental conditions, since we paid special care to avoid degradation of this compound. The effects of DIDS at high concentration (2 mM) with longer exposure time appeared not to be related to inhibition of I_Clswell, but to some non-specific actions such as binding to free amino groups [26]. Therefore, effects of DIDS on membrane potentials and currents during hypotonic challenge are equivocal. On the other hand, another I_Clswell blocker, 9-AC showed distinct effects on these phenomenon. The percentage of cells with activated I_Clswell fits well with the percentage of cells in which 9-AC reversed shortening of APD (about 40%) and is in accordance with literature data [7,15].

Taken together, our results indicate that in about 50% of cells shortening of APD is not due to specific current modulation, but probably to direct effects of hypotonic stress, as discussed below.
4.3. Depolarization of RP

Despite combined application of ouabain, Gd\(^{3+}\), 9-AC and chromanol, depolarization of RP was still observed in hypotonic solution, excluding the contribution of the pump current, NSC, \(I_{\text{Clswell}}\) and \(I_{Ks}\). Although changes in \(I_{Ks}\) are the most prominent factor for determining RP, the most probable candidate for observed depolarization, increased inward current at voltages negative to reversal potentials as well as changes in negative slope in I-V curve and no ‘cross-over phenomenon’ in increased [K\(^+\)]\(_i\) [27] argue against involvement of this current. Parallel shifts in background I-V curves were observed with similar degrees of shifts in reversal potentials and in depolarization of RP with increasing [K\(^+\)]\(_i\). Therefore, we assume that dilution of intracellular ion strength [22], could be a mechanism of this phenomenon, and it might affect action potential repolarization, as well. This is, however, only indirect evidence, and other factors such as changes in permeability ratio for K\(^+\) and Na\(^+\) might develop during hypoosmotic challenge. Exploration of other mechanisms for this phenomenon is to be further done.

4.4. Limitations of the study

This study and most other reports examined electrophysiological effects of hypotonic solution in cardiac myocytes used the standard whole-cell patch-clamp technique. However, in this case, spontaneous cell swelling in isotonic solution due to cell dialysis by pipette solution, especially in atrial myocytes [1], should be taken into account. Also, we did not measure and correlate changes in cell volume with changes in membrane potential and ionic currents. The aim of this study was to find out current background for membrane potential changes, and not to explore the mechanisms of current activation in hypotonic solution. Nevertheless, in order to minimize changes in cell volume in isotonic solution and to achieve steady-state we used slightly hypotonic pipette solution compared to isotonic bath solution, and recorded data at least 7–10 min after whole-cell configuration was established.

This study demonstrated that activation of NSC produced initial prolongation of APD, while activation of 9-AC-sensitive \(I_{\text{Clswell}}\) and activation of \(I_{Ks}\) participated in secondary shortening of APD at least in about 40% of myocytes. Direct effect of hypotonic challenge, as dilution of intracellular ion strength, might be a main reason for depolarization of RP and could probably participate in later shortening of APD to some extent.

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