Increase of the Delayed Rectifier $K^+$ and $Na^+-K^+$ Pump Currents by Hypotonic Solutions in Guinea Pig Cardiac Myocytes

Norihito Sasaki, Tamotsu Mitsuiye, Zhuren Wang, Akinori Noma

Abstract To investigate the membrane current changes induced by membrane stretching, single guinea pig ventricular myocytes were superfused with solutions of various osmolarities, and the whole-cell current was recorded by the patch-clamp technique. The application of 70% and 130% osmolar bath solutions increased and decreased the amplitude of delayed rectifier $K^+$ current ($I_{K}$), respectively, whereas no obvious change was observed in the $L$-type $Ca^{2+}$ current or the inward rectifier $K^+$ current. When the $Na^+-K^+$ pump current ($I_{pump}$) was recorded by the use of high-$Na^+$ (>35 mmol/L) pipette solutions, $I_{pump}$ was also increased and decreased by the superfusion of hypotonic and hyperosmotic solutions, respectively, in approximately half of the cells. An increase of the $I_{pump}$ was also observed in the absence of external $Na^+$, excluding a possibility that the enhancement of $I_{pump}$ was secondary to an elevation of cytosolic $Na^+$. In most cells that did not show the increase of $I_{pump}$, the hypotonic superfusion induced a gradual activation of $Cl^-$ current. The hypertonic superfusion did not cause any consistent change in the membrane $Cl^-$ conductance. Since the response of $I_K$ was observed in all experiments, its mechanism was studied. We failed to observe marked changes in the kinetic and conductance properties of $I_K$ in the hypotonic solution. The involvements of either the protein kinases or $Ca^{2+}$ were also ruled out as major mechanisms underlying the $I_K$ response. (Circ Res. 1994;75:887-895.)

Key Words • cardiac myocytes • cell swelling • delayed rectifier $K^+$ current • $Na^+-K^+$ pump current

The heart rate increases in response to an increase of venous return, and an acceleration of the spontaneous rhythm was demonstrated by stretching the isolated cardiac pacemaker tissue.1,2 Contraction of the ventricle increases under conditions of volume overload.3 These autoregulatory responses to mechanical stimuli are considered to be mediated at least in part by changes in the membrane current. Voltage-clamp analysis, however, is made difficult by a mechanical dislodgment of the intracellular electrode during stretch of the myocyte. To circumvent this difficulty, cell swelling by superfusing hypotonic solutions4 or by applying positive pressure into the whole-cell patch-clamp electrode3 was used to study the effect of stretching the cell membrane on ionic currents. Until now, it has been demonstrated that various types of $K^+$ and $Cl^-$ currents are increased by applying hypotonic solutions or positive pressure in different cell types (sinoatrial nodal cell,5 canine ventricular myocytes,4 human intestinal epithelial cells,6 and lymphocytes7). Our previous study6 compared responses of single ventricular cells between mechanical stretching and hypotonic superfusion. The superfusion of the cell with a hypotonic solution increased the amplitude of the delayed rectifier $K^+$ current ($I_K$) without any marked changes in the time-independent currents. Mechanical stretch applied to the longitudinal axis of the cell, however, occasionally increased the amplitude of a time-independent current, which was most probably mediated by an increase in the intracellular $Ca^{2+}$. It was suggested that cell swelling in the hypotonic solution might reflect the effect of stretching the cell membrane on ionic channels and that the mechanical stretch along the cellular longitudinal axis failed to apply effective stress to the surface membrane.

The present study aims at clarifying changes in the membrane current during superfusion of the ventricular myocytes with hypotonic solutions. It was observed that the amplitude of $I_K$, the $Na^+-K^+$ pump current ($I_{pump}$), and a time-independent $Cl^-$ current ($I_{Cl}$) were increased, whereas the inward rectifier $K^+$ current ($I_{K}$) and the $L$-type $Ca^{2+}$ current ($I_{Ca}$) were not affected. If the myocytes were superfused with hypertonic solutions, both $I_K$ and $I_{pump}$ were depressed. Mechanisms of the osmotic responses of $I_K$ were also investigated, and the significance of these current responses will be discussed.

Materials and Methods

Single ventricular cells were obtained by treating guinea pig hearts with collagenase as previously described.9,10

Solutions

The control Tyrode's solution contained (mmol/L) NaCl 140, KCl 5.4, CaCl$_2$ 1.8, MgCl$_2$ 0.5, NaH$_2$PO$_4$ 0.3, glucose 5.5, and HEPES 5.0. According to Tseng,4 the osmolarity of Tyrode's solution of almost comparable composition is 290±20 mOsm/L and can be varied roughly in proportion to the total amount of salts. The pH of the external and internal solutions was adjusted to 7.4 and 7.2 with NaOH and KOH or CsOH, respectively.

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Solutions Used to Record $I_K$

The composition of the hypotonic (70%) solution used to record $I_K$ was (mmol/L) NaCl 100, NaH$_2$PO$_4$ 0.3, KCl 5.4 or 0, CaCl$_2$ 0.9, MgCl$_2$ 0.5, NiCl$_2$ 2.0 or nicardipine 2 μmol/L, glucose 5.5, and HEPES 5.0. The isotonic solution of comparable ionic compositions was prepared by adding 80 mmol/L mannitol to the hypotonic solution. The hypertonic (130%) solution was prepared by adding 90 mmol/L mannitol in the control Tyrode’s solution, and the membrane currents were compared between these two solutions after blocking the Ca$^{2+}$ currents. In some experiments, a protein kinase inhibitor, 1-(5-isouquinolinylsulfonfonyl)-2-methylpipеразин dihydrochloride (H-7), was added to these external solutions. The composition of the internal solution was (mmol/L) potassium aspartate 140, MgCl$_2$ 5.0, K$_2$-ATP 5.0, phosphocreatine (Na$_2$-PCr) 5.0, EGTA 5.0 or BAPTA 10, and HEPES 5.0 (pH 7.4 with KOH).

Solutions Used to Record $I_{pump}$ and $I_{Cl}$

The composition of the hypotonic solution used to record $I_{pump}$ and $I_{Cl}$ was (mmol/L) NaCl or choline chloride 100, KCl 5.4, MgCl$_2$ 1.8, BaCl$_2$ 0.9, CsCl 1.0, CdCl$_2$ 0.1, glucose 5.5, and HEPES 5.0 (pH 7.4 with KOH). The isotonic solution was prepared by adding 80 mmol/L mannitol or 40 mmol/L NaCl to the hypotonic solution. In preliminary experiments, the amplitude of $I_{pump}$ was not affected by replacing 30% NaCl in the control Tyrode’s solution with mannitol. The hypertonic solution was prepared by adding mannitol to this isotonic solution. Ouabain (20 μmol/L) and anthracene-9-carboxylate (9-AC, 100 to 500 μmol/L, Wakо) were used in these external solutions, if necessary, to block $I_{pump}$ and $I_{Cl}$, respectively. The control internal solution contained (mmol/L) CsOH 90, aspartate 100, NaH$_2$PO$_4$ 1.0, MgCl$_2$ 5.0, tetraethyammmonium chloride 20, EGTA 10, Mg-ATP 10, Na$_2$-PCr 5.0, and HEPES 10. The high Na$^+$ internal solutions were prepared by raising [Na$^+$] to 35, 45, and 110 mmol/L by replacing CsOH with equimolar NaOH.

Voltage-Clamp and Recording Techniques

Single ventricular cells were voltage-clamped using the whole-cell configuration of the patch-clamp technique. The glass suction pipette had a tip diameter of ~3 μm and a resistance of 1 to 3 MΩ when filled with the internal solution. The series resistance was ~8 MΩ, as examined from the time course of the capacitive current recorded at the start of the whole-cell voltage clamp. After the formation of a gigahm seal, a strong suction was briefly applied to rupture the patch membrane. The current and voltage signals were stored on a digital magnetic tape (RD101, TEAC) for later computer analysis (PC98, NEC). The liquid junction potential between the pipette solution and the external solution (~10 mV) was corrected for all membrane potential recordings.

The time- and voltage-dependent currents were examined by applying square pulses from the holding potential of ~40 mV. The time-independent currents, such as $I_{Cl}$ and $I_{pump}$, were investigated by determining the current-voltage (I-V) relation using ramp pulses. A holding potential of 0 mV was used in a part of experiments examining $I_{Cl}$. The ramp pulse was of a sawtooth configuration, and its dV/dt was 2 V/s. $I_{K}$ and $I_{Cl}$ were blocked by replacing K$^+$ with Cs$^+$ and $I_{pump}$ with nicardipine or Ni$^{2+}$, if necessary. In the nonstationary fluctuation analysis of $I_K$, the current signal was digitized at 3.3 kHz through the active filter of 1.5 kHz cutoff frequency.

The exchange of bath solution was performed by switching the perfusates at the inlet of the recording chamber and took ~15 seconds, as examined by the suppression of $I_{K}$ on the removal of external K$^+$.

Data are given as mean±S.D. All experiments were carried out at 35±0.5°C.

Fig 1. Effects of superfusing ventricular myocytes with 70% hypotonic solution on the whole-cell membrane current. A, Chart recording of the membrane current change induced by the 70% hypotonic (Hypo) solution bracketed by the isotonic (Iso) superfusion. Vertical deflections were due to repetitive depolarization to +30 mV for 1 second in every 10 seconds. The holding potential was ~40 mV. The external solution was the K$^+$- and Ca$^{2+}$-free solution. B, Recordings of the L-type Ca$^{2+}$ current ($I_{Ca}$) at 0 mV and the delayed rectifier K$^+$ current at +40 mV in the Iso and Hypo K$^+$-free solutions. The dotted line indicates a zero current level. C, Current-voltage relations measured at the peak $I_{Ca}$ or at ~10 milliseconds into the test pulse (closed symbols) and those measured near the end of the 300-millisecond pulse (open symbols). The circles indicate myocytes in the Iso solution; the triangles, myocytes in the Hypo solution. This experiment is the same as in panel B.

Results

Increase of $I_K$ in Hypotonic Solution

$I_K$ was suppressed by superfusing myocytes with the K$^+$-free solution, and the effects of hypotonic superfusion were investigated on $I_{Ca}$ and $I_K$. The amplitude of $I_K$ started to increase soon after the application of the 70% hypotonic solution to reach a new steady level within 50 seconds as shown in Fig 1A. The recovery of $I_K$ took almost the same time course after switching back to the isotonic solution. In four experiments, the half-time for the enhancement and recovery were 22±2 and 20±3 seconds, respectively.

Fig 1B shows the representative current recordings obtained with depolarizing pulses to 0 and +40 mV in the isotonic solution (left) and ~70 seconds after the application of the hypotonic solution (right). It is evident that the amplitude of $I_{Ca}$ was not affected by the 70% hypotonic solution. On the other hand, the amplitude of $I_K$, measured as a time-dependent increase at +40 mV or the tail current on repolarization, increased remarkably in the hypotonic solution. Fig 1C shows the I-V relations of the initial and late currents. The I-V relations for the peak inward currents in the isotonic (●) and hypotonic (▲) solutions suggested that the gating kinetics and the conductance for $I_K$ were not modified by the intervention. In the I-V curve for late currents, the amplitude of the outward current in the
hypotonic solution (△) is obviously larger than that in the isotonic solution (○) over the range more positive than −10 mV. The I-V relations at potentials more negative than −40 mV were almost superposable. Essentially the same findings were obtained in two other experiments.

The increase of I_k was observed in all experiments. When the amplitude of I_k was measured as the time-dependent current during the pulse from +30 to +50 mV, it was increased to 178±47% (n=7) of the control value at 5.4 mmol/L [K+]o, and to 217±41% (n=9) at 0 mmol/L [K+]o, by the hypotonic solution. Similarly, the saturatory amplitude of the I_k tail current recorded on repolarization to −40 mV was increased to 175±69% (n=4) at 5.4 mmol/L [K+]o, and 191±50% (n=9) at 0 mmol/L [K+]o.

Effects of Hypotonic Solution on I_{k1}

I_{k1} was scarcely affected. In the experiment shown in Fig 2, the membrane currents were recorded by the pulse protocol indicated in the inset after blocking I_{Ca} with 2 mmol/L Ni^{2+}. It is evident that I_{k1} recorded with hyperpolarizing pulses was hardly affected when I_{Ca} was markedly increased by applying the 70% hypotonic solution (right) for ~120 seconds. The initial I-V relation before (○) and during (△) the hypotonic solution confirmed the inactness of I_{k1}. In six experiments, the slope conductance between −70 and −90 mV in the hypotonic solution was 104±14% of the control value.

Decrease of I_k in Hypertonic Solution

To determine the dynamic range of osmotic response of I_k, the myocytes were superfused with the hypertonic (130%) solution. The amplitude of I_k, when activated by depolarizing pulses to +40 mV, showed a marked decrease after switching to the hypertonic solution, as shown in Fig 3A. Reapplication of isotonic solution recovered the amplitude of I_k. In the recordings of the expanded time scale in Fig 3B, the initial inward jump of the current on depolarization may be due to the strong inward rectification of I_k, and the entire time-dependent change might be solely due to the activation of I_k. In this experiment, the amplitude of I_k decreased to 45% of the control value 20 seconds after the hypertonic superfusion. In the same experiment, the hypertonic solution only slightly decreased the amplitude of I_{Ca} recorded by the depolarizing pulse to 0 mV. In all of five experiments, the amplitude of I_k at +30 or +40 mV decreased by 44±2% after a 20-second perfusion of the hypertonic solution. In these experiments, no consistent change of I_{Ca} was observed in the hypertonic solution.

Enhancement of I_{pump} in Hypotonic Solution

In the above experiments using a 10 mmol/L Na^{+} pipette solution, no obvious change was noticed in the amplitude of the time-independent current components. However, the Na^{+}-K^{+} pump may respond to a change in the cellular volume in the hypotonic solution. To examine I_{pump}, the Ca^{2+} and K^{+} conductances were blocked, and the recording of I_{pump} was facilitated by enlarging I_{pump} using the high-Na^{+} pipette solution. The membrane conductance was measured by using ramp pulses. The external application of the hypotonic solution (Fig 4A) shifted the holding current outwardly at −40 mV, and a new steady current level was attained within ~30 seconds. The outward shift of the holding current was reversed by washing out the hypotonic solution, and this response was reproducible.
Fig 4B shows the I-V relations in the control (●) and hypotonic (▲) solutions. In the control condition, the reversal potential of the net membrane current was near −100 mV because of the enhancement of the Na⁺-K⁺ pump activity by the high-Na⁺ pipette solution. The hypotonic solution shifted the I-V curve outwardly over the entire potential range examined, so that the two I-V curves did not cross over. This finding indicates that the current change caused by the hypotonic solution is not attributable to any ionic membrane conductance but suggests an involvement of the Na⁺-K⁺ pump. To confirm whether or not the Ipump is actually activated by hypotonic stimulation, effects of ouabain were investigated. As shown in Fig 4A, the application of ouabain caused a marked inward shift of the holding current, suggesting a large contribution of the Na⁺-K⁺ pump. After washing out ouabain, the holding current gradually returned. In other experiments (n=7), it was observed that the application of hypotonic solution in the presence of ouabain failed to induce the outward shift of the whole-cell current.

To quantify the enhancement of Ipump, the current recorded in the presence of ouabain was subtracted from those recorded in each experimental condition to obtain the ouabain-sensitive current. The I-V relations in Fig 4C indicate the basal Ipump (lower curve) and the enhanced Ipump (upper curve) in the hypotonic solution. The enlargement of Ipump to 166±26% of the control value at 0 mV was observed in 6 of 14 experiments using the 35 to 45 mM Na⁺ pipette solution. When [Na⁺] was 10 mM/L in the pipette solution, the increase of Ipump could be resolved in only 1 of 10 experiments (27% increase). The activation of Ipump started without delay and became saturated within 50 to 150 seconds after switching to the hypotonic solution.

To exclude the possibility that the elevation of Ipump was secondary to an increase of an unknown membrane Na⁺ conductance and a rise in the cytosolic [Na⁺], the whole-cell current was recorded by using both the Na⁺-free external solution and the high-Na⁺ (45 mM/L) pipette solution. Ipump was increased by hypotonic stimulation to 172%, 194%, and 156% of the control value in 3 of 15 experiments. Thus, a sodium influx, if any, is not required for the activation of Ipump in the hypotonic solution.

The Na⁺ is provided to the cell through diffusion from the pipette solution and pumped out by the Na⁺-K⁺ pump.14 We examined whether this diffusion of Na⁺ from the pipette to the cell is a rate-limiting step in those cells that did not show a significant enhancement of Ipump. To maximize the diffusion of Na⁺, the pipette [Na⁺] was increased to 110 mM/L, and essentially the same protocol was repeated. The application of ouabain revealed a large amplitude of Ipump in these experiments, mostly >200 pA at the holding potential. An increase of Ipump was observed in 6 of 8 experiments.

Reduction of Ipump in Hypertonic Solution

To determine the dynamic range of the response of Ipump to varying the osmolarity, changes of Ipump were recorded in the hypertonic solution. 9-AC (500 μmol/L), a partial blocker of the swelling-induced Cl⁻ current, was added to all solutions to minimize possible contamination of Icl. A representative experiment is shown in Fig 5, where chart recording of the current at a holding potential of −40 mV is shown in panel A and the I-V relations at different conditions are shown in panel B. At the beginning of the recording, the reversible increase of Ipump in the hypotonic solution was recorded (● compared with ○ and ▲). Then, the isotonic solution with 80 mM/L mannitol was switched to that without mannitol (140 mM/L Na⁺) to confirm that the amplitude of Ipump was not significantly changed, as shown in Fig 5B (graph b). When the external solution was made hypertonic by adding mannitol to the isotonic solution, the membrane current was largely decreased over the entire potential range examined (▲, Fig 5B [graph c]). Last, the background current was obtained by applying ouabain (■). It is evident that the amplitude of ouabain-sensitive current (Ipump) was markedly decreased by the hypotonic solution. The continuous inward shift of the holding current might be most possibly due to a rundown of Ipump. The depression of Ipump was observed in 15 of 29 experiments.

In the experiment shown in Fig 5, the superfusion of the hypotonic solution increased the amplitude of Ipump by 33% of the control value; the hypotonic solution reduced the current by 79%. In two other experiments in the presence of 9-AC, Ipump was increased by 53% and 42% of the control value in the hypotonic solution, whereas it was reduced by 66% and 72% of the control in the hypertonic solution. Thus, it was concluded that the amplitude of Ipump changes dramatically over an osmotic range including the physiological level.

Enhancement of ICl by Hypotonic Solution

In the canine cardiac myocytes, an increase of the time-independent Cl⁻ conductance was reported in
hypotonic solution. In the present study, the increase of \( I_C \) was observed when the high-\( Na^+ \) (35 to 45 mmol/L) pipette solution was used. As shown in Fig 6A, the holding current at 0 mV gradually shifted outwardly with a delay of \( \approx 20 \) seconds after switching to the hypotonic solution, accompanied with a marked increase in the membrane conductance. On washing out the hypotonic solution, these responses disappeared. The second application of the hypotonic solution induced a similar outward shift of the holding current, but the time course of activation was accelerated.

The increased current was attributed to a \( Cl^- \) conductance by measuring I-V relations (Fig 6B). In the control solution, the current was outward over the potential range examined because of the activation of \( Na^+-K^+ \) pump by the 45 mmol/L \( Na^+ \) pipette solution. In this experiment, however, it is evident that the hypotonic stimulation failed to enhance \( I_{\text{pump}} \). The reversal potential remained at \( \approx -40 \) mV during the gradual increase in the slope conductance, indicating that the current change is based explicitly on an ionic conductance, which is considered to be selective for \( Cl^- \). In three experiments, the reversal potential was \(-40, -41, \) and \(-43 \) mV, respectively (\( Cl^- \) reversal potential, \(-35 \) mV). This membrane conductance was partially blocked by 100 \( \mu \)mol/L 9-AC, a \( Cl^- \) channel blocker. Essentially the same result was observed in 8 of 14 experiments using the high-\( Na^+ \) pipette solution (35 to 45 mmol/L) but in none of the 10 experiments using the 10 mmol/L \( Na^+ \) pipette solution.

The Table examines the correlation between the enhancement of \( I_{\text{pump}} \) and the increase of \( Cl^- \) conductance in the hypotonic solution in experiments using the high-\( Na^+ \) (35 to 45 mmol/L) pipette solution and 100 or 0 mmol/L [\( Na^+ \)]o. The presence of the \( I_{\text{pump}} \) response was accepted by an increase of \( >30\% \) of the control \( I_{\text{pump}} \); the \( I_C \) response, by an increase of \( >0.2 \) nA at 0 mV. It is evident that most of the cells that responded to the hypotonic superfusion by increasing either \( I_{\text{pump}} \) or \( I_C \)

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<th>Enhancement of ( I_{\text{pump}} )</th>
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\( I_{\text{pump}} \) indicates \( Na^+-K^+ \) pump current; \( I_C \), time-independent \( Cl^- \) current. The number of experiments in which the response of \( I_C \) or \( I_{\text{pump}} \) was either observed (+) or not observed (-) using 35 to 40 mmol/L \( Na^+ \) pipette solution is shown. Numbers in parentheses indicate experiments in the \( Na^+ \)-free external solution.
Mechanism of \(I_K\) Enhancement in Hypotonic Solution: Gating Parameters

Since the increase of \(I_K\) by the hypotonic solution was consistently observed in the present study, the mechanism for this response was analyzed. The amplitude of \(I_K\) is determined by the product of the total number of channels, the unit amplitude of single channel current, and the open probability of the channel. At first, the gating kinetics of \(I_K\) was examined. In Fig 7, the time constants of activation (panel A) and deactivation (panel B) were measured. \(I_K\) was elicited by a depolarizing pulse to +60 mV from a holding potential of -40 mV. As described in previous studies, \(I_K\) showed a sigmoidal onset, and its amplitude did not reach a steady level even if the pulse duration was prolonged to 10 seconds. The amplitude of time-dependent change of \(I_K\) was fitted to a sum of two exponentials. The values of fast and slow time constants for activation were 190 and 1219 milliseconds, respectively, in the control solution and 170 and 1412 milliseconds, respectively, in the hypotonic solution; those for deactivation on repolarization were 161 and 925 milliseconds, respectively, in the control solution and 155 and 927 milliseconds, respectively, in the hypotonic solution. The same measurements were made at different potentials, and the data obtained in five experiments are summarized in Fig 8. No marked difference was observed in the measurements of time constants for data obtained in the isotonic and hypotonic solutions.

The quasi–steady-state degree of activation was measured by applying depolarizing pulses of 3 seconds in duration and by measuring the amplitude of tail currents at -40 mV. The voltage relations of the tail amplitude in the isotonic and that in the hypertonic solutions were fitted to the Boltzmann curve as shown in Fig 9. The values of the slope factor were 10±2 and 11±1 mV in the isotonic and hypotonic solutions,
respectively, and potentials for the half-activation were 15±2 and 10±2 mV, respectively (n=4). $I_K$ recorded at potentials (>±50 mV) of the maximum activation was enlarged by the hypotonic solution, which suggests that the slight change in the gating mechanisms is not the major cause for the increase of $I_K$ in the hypotonic solution.

**Conductance Parameters of $I_K$ in Hypotonic Solution**

The elementary amplitude of $I_K$ was measured by conducting the ensemble noise analysis. A number (n=8 to 20) of consecutive recordings of $I_K$, activated by an identical pulse, were digitized after a low-pass filter (1.5 kHz), and mean±variance ($\sigma^2$) was calculated for n digital points at the same sampling time, step by step over the entire pulse duration. The variance-mean relation in Fig 10 was fitted to the following binomial distribution:

$$\sigma^2 = N \cdot \frac{P_e}{1-P_e} i^2 = i(1-P_e)I$$

where N is the number of channels, $P_e$ is open probability, i is the unit amplitude of the current, and I is the mean current amplitude, given as $\text{INP}_e$. In both the isotonic and the hypotonic solutions, the relations were well fitted by Equation 1, with i and N of 0.71 pA and 20 237 at +50 mV, respectively. In other experiments, they were 0.70 pA and 32 013 at +50 mV, respectively. In three other experiments at +30 mV, the averages were 0.60 pA and 24 800, respectively. Although the values of N were not significant because of limited amplitude of current recorded, it was concluded that the amplitude of single-channel current, which was determined by the initial slope of the relation, was not modified in the hypotonic solution.

In agreement with the above view, no obvious change was observed in the reversal potential of $I_K$. In the experiment shown in Fig 11, the $I_K$ tail reversed its direction near −70 mV in both the isotonic and hypotonic solutions. The reversal potentials at 5.4 mmol/L $[K^+]_o$, when determined from the crossing of the two I-V curves for the initial and late currents (not shown), were −66±3 mV in the control solution and −60±10 mV (n=4) in the hypotonic solution. Those measured at 0 mmol/L $[K^+]_o$ were −100±11 mV in the control solution and −108±5 mV in the hypotonic solution (n=6).

**Other Mechanisms to Modulate $I_K$**

Regulatory mechanisms of $I_K$ described in the previous studies, such as β-adrenergic stimulation,15,17 phorbol ester stimulation,18,19 and the elevation of $[Ca^{2+}]$,20 were examined regarding the effect of the hypotonic solution observed in the present study. The external application of H-7 (100 μmol/L), a nonspecific inhibitor of protein kinases, failed to block the hypotonic response of $I_K$ (not shown). Isoprenaline (<2 μmol/L) increased the amplitude of $I_K$ in the control condition, and the hypotonic superfusion further increased the amplitude. The hypotonic response of $I_K$ was also observed when an elevation of $[Ca^{2+}]$ was prevented by the use of both 10 mmol/L BAPTA in the pipette solution and a Ca$^{2+}$-free external solution. It might be
suggested that the hypotonic response of $I_K$ was not mediated by the protein kinases or an elevation of $[\text{Ca}^{2+}]$.

**Discussion**

The present study demonstrated that $I_K$, $I_{\text{pump}}$, and $I_{\text{Cl}}$ were increased reversibly by superfusing ventricular myocytes with hypotonic solution. Essentially the opposite responses were observed for $I_K$ and $I_{\text{pump}}$ by superfusion of the hypertonic solution, which suggests that these currents respond to the change in osmolarity around the physiological level. We failed to observe any current changes that might be due to a change in the ATP-sensitive $K^+$ current$^{21}$ or the nonspecific cation current$^{22}$ in various osmotic solutions used in the present study.

Since the response of $I_K$ was observed in every cell examined, the mechanism for this response was extensively studied. We failed to observe marked changes in the kinetic and conductance properties of $I_K$ in the hypotonic solution. The ventricular $I_K$ is separated into E-4031-sensitive ($I_{\text{Ks}}$) and insensitive ($I_{\text{Ku}}$) components by Sanguinetti and Jurkiewicz.$^{23}$ Thus, it might be asked which of these components is responsible for the hypotonic increase of $I_K$ in the present study. The increase of $I_K$ was also observed at positive potentials such as +40 mV, where E-4031 is ineffective. Therefore, $I_{Ku}$ might be increased by the hypotonic solutions. The first exponential component of the tail current showed time constants comparable to those of $I_{Ks}$, and it was increased by the hypotonic solution (Fig 7B). This result may suggest that $I_{Ku}$ was also increased by the hypotonic solution. The involvement of either the protein kinases or Ca$^{2+}$ described in the previous studies$^{17,20}$ was also ruled out from the major mechanisms underlying the $I_K$ response. One obvious possibility, which remained unexamined, was the increase in the number of available channels induced directly by the stretching of the cell membrane.

No quantitative measurement of the strength of stretch applied to the membrane in the hypotonic solutions is available in the present study. However, the response of $I_K$ to the external osmolarity change developed soon after switching the solution and became saturated within 30 seconds. This was in contrast to the response of $I_{\text{Cl}}$, which was delayed and progressive during the hypotonic superfusion. Different time courses of activation between $K^+$ and $Cl^-$ conductances were also reported in the intestinal epithelial cells.$^6$

The enhancement of $I_{\text{pump}}$ by the superfusion of the hypotonic solution took a time course similar to or slightly slower than the response of $I_K$. The response started without delay and became saturated to $\approx$160% of the control value within 50 to 150 seconds after switching to the hypotonic solution. We excluded the possibility that the pump enhancement was secondary to an accumulation of the cytosolic $\text{Na}^+$, since the response could be observed in the absence of the external $\text{Na}^+$. Thus, the stimulation of the $\text{Na}^+-\text{K}^+$ pump directly by the stretch of the membrane might be expected.

In contrast to the $I_K$ response (100%), the increase of $I_{\text{pump}}$ was observed only in 45% and 75% of the experiments, when the pipette $[\text{Na}^+]$ was raised to 35 to 45 mmol/L and 110 mmol/L, respectively. However, it is still possible that the $\text{Na}^+-\text{K}^+$ pump was potentiated without any obvious increase of the recorded $I_{\text{pump}}$ in the hypotonic solution. In the experiments in which no obvious increase in $I_{\text{pump}}$ was observed, the supply of $\text{Na}^+$ from the pipette by diffusion might be limited even in the control condition. This assumption explains well why the enhancement of $I_{\text{pump}}$ was not observed when $[\text{Na}^+]$ was 10 mmol/L in the pipette solution. The use of the 110 mmol/L $\text{Na}^+$ pipette solution may thus override the limitation of diffusion from the pipette tip in activating the $\text{Na}^+-\text{K}^+$ pump.$^{24,25}$

The external application of hypotonic solution reversibly increased the $Cl^-$ conductance. This increase of $Cl^-$ conductance was observed mostly when the elevation of $I_{\text{pump}}$ was marginal (Table) and was progressive during the hypotonic stimulation. The application of the high-$\text{Na}^+$ pipette solution might facilitate the cell swelling in the hypotonic solution, because the osmotic contribution of $\text{Na}^+$ may be larger than that of Cs$^+$, provided that the membrane background conductance is larger for Cs$^+$ than for $\text{Na}^+$.$^{26}$ The failure of the $\text{Na}^+-\text{K}^+$ pump activation in the hypotonic solution might cause an accumulation of $\text{Na}^+$ and facilitate the cell swelling.

In respect to both the outward rectification of the $I_{\text{Cl}}$,voltage relation and the inhibition by 9-AC, $I_{\text{Cl}}$ activated by the hypotonic stimulation in the present study is similar to that recorded in canine cardiac myocytes,$^{4,27}$ rabbit sinoatrial nodal cells,$^5$ and human intestinal epithelial cells.$^6$ In previous studies, $I_{\text{Cl}}$ was also activated by inhibiting the synthesis of cytoskeleton.$^{4,28}$ The increase of $Cl^-$ conductance might be responsible for the regulatory volume decrease, which is reported in cultured heart cells.$^{29}$

Ebara and Hasegawa$^{30}$ observed that the action potential of guinea pig papillary muscle was prolonged in the hypertonic solution and reduced in the hypotonic solution. These findings are well explained by the decrease and increase of $I_K$ observed in the corresponding osmotic solutions. In the experiment using whole-heart preparation, an application of volume overload by the use of a balloon in the left ventricle decreased the action potential duration through an acceleration of phase 3 repolarization.$^{31}$ This finding may also be explained by the change in $I_K$. However, at present we cannot conclude that the increase of $I_K$ is due to the stretching applied to the cell membrane as a result of cell swelling. In the present study, the response of $I_K$ was observed over the range of 70% to 130% of normal osmolarity and was completely reversible. It may thus be concluded that $I_K$ takes the role of osmoelectrical signal transduction in the cardiac ventricular cells.

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