Effects of acute cooling/rewarming on membrane potential and K+ currents in rat ventricular myocytes

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Abstract: The effects of acute cooling/rewarming on cardiac K+ currents and membrane potential were investigated. Membrane potential and current were assessed with whole-cell patch-clamp technique in current- and voltage-clamp modes. When the temperature of bath solution was decreased from 25 ºC to 4 ºC, the transient outward current (Ito) was completely abolished, the sustained outward K+ current (Iss) at +60 mV and the inward rectifier K+ current (IK1) at -120 mV were depressed by (48.5±14.1)% and (35.7±18.2)% respectively, and the membrane potential became more positive. After the temperature of bath solution was raised from 4 ºC to 36 ºC, the membrane potential exhibited a transient hyperpolarization and then was maintained at a stable level. In some myocytes (36 out of 58), activation of the ATP-sensitive K+ (KATP) channels after rewarming was observed. The rewarming-induced change in the membrane potential was inhibited by the Na+/K+-ATPase inhibitor ouabain (100 μmol/L), and the rewarming-elicited activation of KATP channels was inhibited by the protein kinase A inhibitor H-89 (100 μmol/L). Moreover, decrease of the temperature from 25 ºC to 4 ºC did not induce any significant change in cell volume when the cell membrane potential was clamped at 0 mV. However, significant cell shrinkage with spots was observed soon after rewarming-induced activation of KATP channels. These data demonstrate that acute cooling/rewarming has a profound influence on the membrane potential and K+ currents of ventricular myocytes, and suggest that activation of KATP channels may play a role in cardiac cooling/rewarming injury.

Key words: hypothermia; rewarming; potassium channels; membrane potentials; myocardium

Research Paper

急性低温 / 再复温对大鼠心室肌膜电位和钾电流的影响

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摘 要: 本文旨在研究急性低温 / 再复温对大鼠心室肌膜电位和钾电流的影响。膜电位和膜电流分别在全细胞膜片钳的电压钳和电流钳模式下记录。当细胞外灌流液从 25 ºC 降低到 4 ºC 后, 过性外向电流(transient outward current, Ito)完全消失, 膜电位为 +60 mV 时的稳定外向 K+ 电流(sustained outward K+ current, Iss)和膜电位为 -120 mV 时的内向整流 K+ 电流(inward rectifier K+ current, IK1)分别降低((48.5±14.1)% 和 (35.7±18.2)%, 同时, 膜电位绝对值降低。当细胞外灌流液从 4 ºC 再升高到 36 ºC 后, 膜电位出现一过性超级化, 然后恢复到静息电位水平; 在 58 个细胞中, 有 36 个细胞伴随复温出现 ATP-敏感性 K+ (ATP-sensitive K+, KATP) 通道的激活。再复温引起的上述变化可以被 Na+/K+-ATP 酶抑制剂哇巴因(100 μmol/L)所抑制。再复温引起的 KATP 通道激活也能被蛋白激酶 A 抑制剂 H-89 (100 μmol/L)所抑制。在细胞膜电位被钳制在 0 mV 时, 当细胞外灌流液温度从 25 ºC 降低到 4 ºC 后, 细胞的体积没有发生明显改变, 但当再复温引起 KATP 通道激活后, 细胞很快发生皱缩, 同时细胞内部出现许多折光较强的斑点。上述结果表明急性低温 / 再复温对大鼠心室肌膜电位和 K+ 电流有明显影响, 并提示 KATP 通道激活可能与心肌低温 / 再复温损伤有关。

关键词: 低温; 再复温; 钾通道; 膜电位; 心肌

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In heart transplantation, cold preservation and warm reperfusion are considered important causes of early cardiac graft loss. Although calcium flux, acidosis, ATP depletion, and reactive oxygen species have been defined as putative causative factors, other mediator factors remain to be identified. Indeed, beside necrosis, apoptosis is one of the early events of either warm or cold ischemia/reperfusion injury in the heart.

Recently, more attention has been paid to the activation and/or modulation of specific ionic transport mechanisms including ion channels and ATPases, as mediators of altered intracellular ionic homeostasis leading to depolarization of the plasma membrane potential during apoptosis. 

K⁺ channels play an important role not only in maintaining the resting membrane potential and controlling the excitability of many excitable cells, but also in regulating cell volume and apoptosis. Although it is reported that change in temperature has profound effects on cardiac K⁺ channels including inward rectifier K⁺ current (Iₛᵣ), transient outward K⁺ current (Iₒ) and sustained outward K⁺ current (Iₛₛ) the role of these K⁺ channels in cold ischemia/reperfusion injury is not fully understood.

In the present study, we observed the effect of acute cooling/rewarming on cardiac K⁺ currents, and found that low temperature suppressed Iₛᵣ, Iₒ and Iₛₛ. Rewarming the cardiac myocytes from 4 °C to 36 °C induced activation of ATP-sensitive K⁺ (Kₘᵦₛ) channels, which subsequently resulted in shrinkage of the cell and swelling of subcellular organelles at the plasma membrane potential depolarization condition. These results suggest that rewarming-induced activation of Kₘᵦₛ channels accompanied by decrease of transmembrane potential may be, at least in part, responsible for the cardiac cooling/rewarming injury.

1 MATERIALS AND METHODS

1.1 Cell preparations

Adult Sprague-Dawley (200-250 g) rats of either sex were anesthetized with urethane (1.5 g/kg body weight, i.p.). The chest was opened under artificial ventilation and the aorta was cannulated in situ. The hearts were excised rapidly and retrogradely perfused at 37 °C with the following solutions in turn: Tyrode’s solution (5 min), Ca²⁺-free Tyrode’s solution (5 min), Ca²⁺-free Tyrode’s solution with 0.5 mg/mL collagenase Type II (Gibco, Grand Island, NY, USA) and 1 mg/mL bovine serum albumin (BSA) (35 min), and Kraftbrühe (KB) (high K⁺) solution (5 min). After disassociation and collection, the cells were kept in KB solution at room temperature (23-25 °C) for electrophysiological recordings.

1.2 Whole-cell patch-clamp experiments

Aliquots of cell suspension were transferred into Dagan thermally conductive recording chamber on the stage of a Leica DM IRB inverted microscope (Leica, Germany). The temperature of the recording chamber was controlled by a High Performance Temperature Controller (Dagan Corporation, Minneapolis, MN, USA). Pipettes had tip resistances of 2-2.5 MΩ when filled with internal solution. Whole-cell recordings were performed using an EPC-10 patch-clamp amplifier and Pulse software (HEKA Elektronik, Lambrecht, Germany). The offset potentials between both electrodes were zeroed before the pipette touched the cell. The liquid junction potential between the pipette and the bath solutions was calculated by using the JPCalc program within Clampex 8.1 (Axon Instruments, Inc.) and corrected for each recording. The membrane potential was monitored under the zero-current clamp mode of whole-cell configuration. Iₛᵣ was elicited by 400-ms pulses from a holding potential of -50 mV to test potentials ranging from -30 to +60 or +80 mV in 10-mV step increments. Iₛᵣ was elicited by voltage steps ranging from -120 mV to -30 mV, from a holding potential of -40 mV, during a 400 ms period, with step increments of 10-mV. In most experiments, single cardiac myocytes were voltage-clamped at 0 mV to continuously monitor the effects of temperature change. The current signals were low-pass filtered at 5 kHz and stored in the hard disk of a compatible computer. Since whole-cell membrane capacitance (Cm) is an index of cell size, Cm compensation was performed before and after the recordings to estimate the change in cell size.

1.3 Image capture and digital image analysis

Images were acquired with a Nikon DXM1200F digital camera (Nikon, Japan), connected to the Leica DM IRB inverted microscope during patch-clamp recording and controlled by Nikon ACT-1 software (Media Cybernetics, USA). Cell area was quantitated using Image-Pro Plus (Media Cybernetics, USA).

1.4 Solutions

The Tyrode’s solution contained (in mmol/L): NaCl 143, KCl 5.4, MgCl₂ 0.5, CaCl₂ 1.8, NaH₂PO₄ 0.3, glucose 5, and HEPES-NaOH 5 (pH 7.4). The nominally Ca²⁺-free Tyrode’s solution was made by omitting CaCl₂ from the normal solution. The KB solution contained (in mmol/L): potassium glutamate 70, KCl 25, taurine 20, KH₂PO₄ 10, MgCl₂ 3, EGTA 0.5, glucose 10 and HEPES-KOH 10 (pH 7.35). The standard pipette solution for recording cardiac
K+ currents contained (in mmol/L): K-aspartate 110, KCl 20, MgCl₂ 1, Na₂-phosphocreatine 5, Mg-ATP 5, EGTA 5, and HEPES 10 (pH was adjusted to 7.2 with KOH). The bath solution contained (in mmol/L): NaCl 140, KCl 5, MgCl₂ 1, KH₂PO₄ 0.4, CaCl₂ 1.8, CdCl₂ 0.5, glucose 10, and HEPES-NaOH 5 (pH 7.4). CdCl₂ was used to inhibit the L-type calcium current (I_{Ca,L}) and the Ca²⁺-activated Cl⁻ current. In some experiments, Ba²⁺ (1 mmol/L) was added to the bath solution to block I_{K1}.

1.5 Chemicals
All chemicals were purchased from Sigma (St. Louis, MO, USA). Stock solutions of H-89 (100 mmol/L) and glibenclamide (100 mmol/L) in DMSO was diluted to the desired final concentrations immediately before use. DMSO (≤0.1%) alone did not affect the cardiac membrane currents.

1.6 Statistical analysis
The data were presented as mean±SD. Statistical differences in the data were evaluated by Student’s t-test or ANOVA as appropriate, and were considered significant at values of P<0.05.

2 RESULTS

2.1 Effects of hypothermia on the outward K⁺ currents and I_{K1} in rat ventricular myocytes
Figure 1 showed that change of temperature on the outward K⁺ currents. The present results revealed that I_{to} was much more sensitive to the change of low temperature and was almost completely abolished when the temperature of bath solution decreased from 25 °C to 4 °C (Fig.1B). In contrast, I_{SS} was less sensitive to the drop of temperature than I_{to} (Fig. 1C). I_{SS} at +60 mV was decreased by (48.5±14.1)% after the temperature of chamber was down to 4 °C for 5 min (n=7, P<0.01). We then observed the effect of hypothermia on the I_{K1}. When the temperature of the bath solution was dropped from 25 °C to 4 °C, I_{K1} at -120 mV was reduced from (-2.56±0.64) nA to (-1.60±0.24) nA [(decreased by (35.7±18.2)%), n=6, P<0.01, Fig. 2]. These results are in good agreement with other observations[12,14].

![Fig. 1. Effects of hypothermia on the outward K⁺ currents in rat ventricular myocytes. Outward currents were elicited by 400-ms depolarizing pulses from a holding potential of -50 mV to test potentials ranging from -30 to +80 mV in 10-mV increments. I_{K1} was blocked by 1 mmol/L Ba²⁺. A: Representative current traces recorded at 25 °C (a), after temperature was reduced to 4 °C for 1 min (b) and 5 min (c), and after temperature was returned to 25 °C for 5 min (d). The dashed line indicates level of zero current. B: Current-voltage relationship of I_{to} shown in A (a and c). C: Current-voltage relationship of I_{SS} measured at the end of test pulses for recordings shown in A (a and c). MP, membrane potential. The data in panels A represent seven experiments.](image-url)
Fig. 2. Effect of hypothermia on the inward rectifier K⁺ current (I\(\text{K}1\)) in rat ventricular myocytes. Currents were elicited by pulses from a holding potential of -40 mV for 100 ms to inactivate Na⁺ current and then to 400 ms test potentials ranging from -120 to -30 mV in 10-mV increments. A: Representative current traces recorded at 25 °C (a), 4 °C (b) and after returned to 25 °C (c). The dashed line indicates level of zero current. B: Current-voltage relationship measured at the end of test pulses for recordings shown in A (a and b). MP, membrane potential. The data in panels A represent six experiments.

Fig. 3. Effects of cooling/rewarming on the membrane potential in rat ventricular myocytes. A: Membrane potential was monitored continuously with current clamp technique (upper panel). The lower panel was the outward currents elicited by pulses from a holding potential of -10 mV to 400 ms test potentials ranging from -80 to +80 mV in 10-mV increment, and recorded at different temperatures at the time indicated by the symbol of ‘Λ’ in the upper panel. The dashed line indicates level of zero current. B: Current-voltage relationship measured at the end of test pulses for recordings shown in the lower panel of A. C: Membrane potential was monitored continuously in the presence of 100 μmol/L ouabain. Change of temperature in the upper panel of A and C is indicated by the zigzag line. The data in panels A and C represent five and three experiments, respectively. MP, membrane potential.
2.2 Effects of cooling/rewarming on the membrane potential of rat ventricular myocytes

K⁺ currents are very important for maintaining cardiac resting membrane potential. Since the present data showed that cardiac K⁺ currents was sensitive to the change of temperature, we next observed the effects of cooling/rewarming on the membrane potential (Fig. 3). When the temperature was reduced from 25 °C to 4 °C, the membrane potential became more positive [from (-38.4±12.6) mV at 25 °C to (8.1±16.1) mV at 4 °C, n=5, P<0.01]. After the temperature of bath solution was raised from 4 °C to 36 °C, membrane potential exhibited a transient hyperpolarization with a maximum of (-97.3±5.9) mV and then was maintained at a stable level [(-74.2±10.7) mV, n=5].

To examine the mechanism of temperature-induced change of membrane potential, we observed the effect of cooling/rewarming on the cardiac cells treated with ouabain, a Na⁺/K⁺-ATPase inhibitor. As shows in Fig. 3C, in the presence of 100 μmol/L ouabain, the myocytes was much less sensitive to the rewarming. When the temperature was increased from 4 °C to 36 °C, the membrane potential was only observed a small change [(4.1±10.7) mV at 4 °C vs (-27.1±10.9) mV at 36 °C], which was statistically different from the change in control group (without ouabain treatment) (P<0.05). These results support the view that after hypothermia the membrane potential is directly affected by an electrogenic sodium pump[15]. Moreover, when the temperature was elevated from 4 °C to 36 °C, a significant increase of outward current was observed in 36 out of 58 (62%) myocytes (lower panel in Fig. 3A).

2.3 Effects of cooling/rewarming on membrane K⁺ currents and the configuration of rat cardiac ventricular myocytes

K⁺ channels play an important role in maintaining normal cell volume. To determine whether change of the K⁺ cur-

Fig. 4. Change of cell morphology after rewarming-induced increase of outward K⁺ current. A: The upper panel was a representative current trace showing the change of membrane current at 0 mV. L-type Ca²⁺ current was blocked by Cd²⁺. The lower panel was representative outward current traces, elicited by the protocol described in Fig. 1, recorded at the time indicated by the symbol of ‘ ∧ ’ in the upper panel. The dashed line indicates level of zero current. B: Example images of rat cardiac ventricular myocytes from the cell image collection in the control (25 °C) (left), and rewarming-induced activation of outward K⁺ current for 10 min (right). Scale bar, 10 μm. Change of temperature in the upper panel of A was indicated by the zigzag line. The data in panels A and B represent five and seventeen experiments, respectively.
Fig. 5. Effect of glibenclamide on rewarming-elicited outward current. A and the upper panel in B were the whole-cell current, continuously monitored at 0 mV. Change of temperature in the upper panel of A and B was indicated by the zigzag line. Application of glibenclamide (15 μmol/L) was indicated by horizontal bar. The lower panel in B was representative current traces, elicited by the protocol described in Fig. 1, recorded at the time indicated by the symbol of ‘Λ’ in upper panel. The dashed line indicates level of zero current. The data in panels A and B represent five and seven experiments, respectively.

Fig. 6. Effect of H-89 on rewarming-elicited outward current. The upper panel was the whole-cell current, continuously monitored at 0 mV. Change of temperature in the upper panel was indicated by the zigzag line. Application of H-89 (100 μmol/L) was indicated by horizontal bar. The lower panel was representative current traces, elicited by the protocol described in Fig. 1, recorded at the time indicated by the symbol of ‘Λ’ in upper panel. The dashed line indicates level of zero current. The data represent four experiments.
rents induced cell morphology change, we observed the change of cell shape during the membrane potential was clamp at 0 mV. Decrease of the temperature from 25 °C to 4 °C did not induce any significant change either in membrane current at 0 mV or in cell image. However, with the activation of the outward currents induced by rewarming, significant cell shrinkage with spots all around the cell was observed soon after the activation of the current (Fig. 4). The cell plane sizes at 10 min after occurrence of rewarming-activated outward current was decreased from (2 786.5±1 472.5) μm² to (1 980.19±1 305.89) μm², and the Cm decreased from (113.5±38) pF to (90.4±27.6) pF (Fig. 4, P<0.01, n=17).

2.4 Effects of glibenclamide and H-89 on rewarming-enhanced outward current

To determine the property of rewarming-induced outward current, we observed the effect of glibenclamide, a specific K<sub>ATP</sub> channel inhibitor, on the current. In control, the current elicited by rewarming showed a rapid decay at beginning and then remained relatively stable [(23.1±5.4)μA] of the peak current at 25 min after the activation, n=5, Fig. 5A]. However, the current enhanced by rewarming could be completely inhibited by 15 μmol/L glibenclamide (n=7, Fig. 5B), suggesting that the enhanced current was ATP-sensitive K⁺ current (I<sub>KATP</sub>).

Moreover, temperature stress, a complicated problem that is far from being solved, may involve multiple cell signaling pathways. Since protein kinase A (PKA)-signaling is a key regulator of ion channels, the effect of putative PKA inhibitor H-89 was examined. Figure 6 showed that rewarming-enhanced current was also inhibited by 100 μmol/L H-89 (n=4). This observation indicated that PKA-signaling may be responsible forrewarming-induced activation of K<sub>ATP</sub> channel.

3 DISCUSSION

K⁺ channels play a crucial role in cell function\[10,11\]. It is found that apoptosis is one of the early events of cold ischemia/reperfusion injury\[8\]. Whether cardiac K⁺ channels play a role in the cold ischemia/reperfusion injury is not clear. The present study found that, in agreement with previous studies\[12,14\], low temperature inhibited I<sub>K1</sub>, I<sub>SS</sub> and I<sub>K1</sub>, and decreased the membrane potential. Rewarming could induce a transient hyperpolarization at the initial stage before the membrane potential reached to normal resting potential. The rewarming-induced change in the membrane potential could be largely prevented by the Na⁺/K⁺-ATPase inhibitor ouabain, indicating that an electrogenic sodium pump was involved in this process\[13\]. It is reported that intracellular sodium accumulation during the anoxia period may play a role in anoxia-reoxygenation injury\[9\]. Intracellular sodium accumulation may also exist during hypothermia since there is a decrease in the activity of Na⁺/K⁺-ATPase. It is of interest that rewarming may induce activation of a K⁺ current. Since the current was sensitive to the specific K<sub>ATP</sub> channel blocker glibenclamide, it is likely that the K⁺ current induced by rewarming is I<sub>K<sub>ATP</sub></sub>.

Myocardial sarcolemmal K<sub>ATP</sub> (sarck<sub>ATP</sub>) channel, acting as molecular sensors of cellular metabolism, is inhibited by intracellular ATP\[17\]. Under physiological K⁺ gradient, the reversal potential of K<sub>ATP</sub> channel is around -80 mV estimated from the Nernst equation. If the membrane potential is positive to the reversal potential, activation of sarck<sub>ATP</sub> channel will induce a K⁺ efflux from the cell and subsequently result in loss of intracellular K⁺ and therefore hypoosmotic condition within cell. The relative hyperosmosis of the extracellular fluid and the solution within subcellular organelles may draw the water out of the cardiac myocyte as well as into the organelles. Indeed, the present study found that, when the membrane potential was clamped at 0 mV, cell shrinkage with spots in the cells occurred soon after activation of sarck<sub>ATP</sub> channel. These results indicate that loss of intracellular K⁺ by activation of sarck<sub>ATP</sub> channel impairs the ability of the cardiac myocytes to maintain a stable volume.

The maintenance of normal cell volume is an absolute prerequisite for normal cell function and survival. Sustained cell shrinkage, i.e. apoptotic volume decrease (AVD), is a major hallmark of apoptotic cell death\[18,19\]. Studies have determined that a fundamental movement of ions, particularly monovalent ions, underlies the AVD process and plays an important role in controlling the cell death process. An accumulation of evidence has revealed that enhanced K⁺ efflux has been shown to be an essential mediator of not only early apoptotic cell shrinkage, but also downstream caspase activation and DNA fragmentation\[20\]. Hughes et al’s work suggested that a decrease in intracellular K⁺ concentration is a necessary early event in programmed cell death\[21\]. Moreover, mitochondria play a key role in determining cell fate during exposure to a variety of stress conditions such as ischemia/reperfusion\[22\]. Mitochondrial swelling may cause activation of the apoptotic pathway\[23,24\]. The present result indicated that rewarming-induced activation of K<sub>ATP</sub> channel may induce both the whole-cell shrinkage and the swelling of subcellular organelles, suggesting that activation of sarck<sub>ATP</sub> channel by rewarming may play a role in the apoptosis in cold ischemia/reperfusion injury.
The role of sarcK\textsubscript{ATP} channel in cardiac protection is still controversial\textsuperscript{[25,26]}. Recent evidence indicates that the sarcK\textsubscript{ATP} channel closing, rather than opening, can lead to cardiac protection\textsuperscript{[27,28]} and reduce myocyte apoptosis\textsuperscript{[29]}. As mentioned above, K\textsuperscript{+} influx or efflux through the sarcK\textsubscript{ATP} channel has a close relation with the membrane potential. If the resting potential is more positive to the reversal potential of the K\textsuperscript{+} channel, a continuous loss of intracellular K\textsuperscript{+} will be inevitable. From this point of view, whether sarcK\textsubscript{ATP} channel produces cardiac protection is largely dependent on the resting potential. Since the resting potentials may vary with different experiment conditions, it is not difficult to understand the inconsistent observations concerning the role of sarcK\textsubscript{ATP} channel in cardiac protection.

In summary, temperature-stress has a significant influence on cardiac K\textsuperscript{+} channels. Among the cardiac K\textsuperscript{+} channels, activation of the sarcK\textsubscript{ATP} channel by rewarming may induce markedly change in cell volume when the membrane potential is positive to the reversal potential of the K\textsuperscript{+} current. The role of sarcK\textsubscript{ATP} channel in cooling/rewarming injury should be further investigated.

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