Antiarrhythmic Effect of Acupuncture Pretreatment in Rats Subjected to Simulative Global Ischemia and Reperfusion — Involvement of Adenylate Cyclase, Protein Kinase A, and L-Type Ca^{2+} Channel

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Abstract: Our previous study showed that electro-acupuncture (EA) pretreatment protects the heart from injury of ischemia. The present study explored further whether adenylate cyclase (AC), protein kinase A (PKA), and L-type Ca^{2+} channel, the β_1 -AR signaling components modulating intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$), are involved in the mediation of the antiarrhythmic effect of EA pretreatment in the rats from which the hearts were subsequently isolated and subjected to simulative global ischemia and reperfusion (SGIR). SGIR was performed by perfusing the isolated heart at a low flow followed by normal perfusion. Adult rats were randomized into four groups, namely, normal control (NC), SGIR, EA, and NC plus EA (NCEA) groups. The rats in the EA and NCEA groups were given EA pretreatment at bilateral Neiguan points (PC6) for 30 min once a day in 3 consecutive days before the hearts were isolated

and perfused. The arrhythmia score and the response of $[\text{Ca}^{2+}]_i$ to the activators of AC, PKA, and L-type Ca^{2+} channel in single ventricular myocyte isolated from the hearts subjected to SGIR were compared among the groups. The results showed that the arrhythmia score was significantly higher in the SGIR group as compared with the NC and NCEA groups. The SGIR-enhanced arrhythmia score was significantly attenuated in the EA group. More interesting, EA pretreatment also attenuated the SGIR-enhanced response of $[\text{Ca}^{2+}]_i$ to the activators of AC, PKA, and the L-type Ca^{2+} channel in the myocytes isolated from the hearts subjected to SGIR. In conclusion, EA pretreatment can produce an antiarrhythmic effect in the rat of SGIR. AC, PKA and the L-type Ca^{2+} channel are involved in the mediation of the antiarrhythmic effect of EA pretreatment.

Key words: electro-acupuncture pretreatment, arrhythmia, adenylate cyclase, protein kinase A, L-type Ca^{2+} channel.

Arrhythmia is one of the most typical clinical manifestations in patients with cardiac ischemia and reperfusion. It is well documented that overactivation of the sympathetic nervous system is an important factor in the genesis of ventricular arrhythmias in patients with impaired ventricular function [1]. It is well known that β_1 -adrenoceptor (β_1 -AR), which acts as the most powerful cardiac receptor, is stimulated by the sympathetic neurotransmitters. The activation of cardiac β -adrenoceptors (β -ARs) by the agonists of β -ARs, including isoproterenol and norepinephrine, causes the arrhythmias [2, 3]. The wide use of β_1 -AR blockers in the treatment of arrhythmias [4] further confirms the involvement of β_1 -AR in the genesis of cardiac arrhythmias.

Similar to the cardioprotection produced by ischemic preconditioning, repetitive stimulation to either β_1 -AR

or the components of its signaling pathway before myocardial ischemia and reperfusion (MIR) could attenuate the myocardial injury induced by the following prolonged ischemia and reperfusion [5–7]. Thus repetitive pretreatment with electro-acupuncture (EA), which was shown to excite the sympathetic nervous system, may also produce the cardioprotective effect via influencing β_1 -AR and/or its signaling pathway. The results of our previous study showed that pretreatment with EA at bilateral Neiguan acupoints protects the heart from the injuries of ischemia and reperfusion via the inhibition of cardiac β_1 -AR and Gs-protein [8]. Since arrhythmias are the major manifestation of cardiac injury induced by ischemia and reperfusion, EA pretreatment may also ameliorate MIR-induced arrhythmias by blunting the β_1 -AR signaling. The previous study showed that the

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intracellular calcium is related to the arrhythmogenesis [9]. During the MIR, the increase in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) is associated with early afterdepolarization and consequently results in cardiac arrhythmias [10]. The factors affecting cytosolic Ca^{2+} are documented to be involved in the cause of arrhythmias [11, 12]. The purpose of the present study was to determine whether adenylate cyclase (AC), protein kinase A (PKA), as well as L-type Ca^{2+} channel, the β_1 -AR signaling components modulating $[\text{Ca}^{2+}]_i$, are involved in the mediation of the antiarrhythmic effect of EA pretreatment at bilateral Neiguan (PC6) acupoints by measuring the response of $[\text{Ca}^{2+}]_i$ to forskolin, an activator of AC, to 8-Br-cAMP, an activator of PKA, and to Bay K-8644, an opener of the L-type Ca^{2+} channel in the single ventricular myocyte isolated from the rats subjected to the simulative global ischemia-reperfusion (SGIR). It was shown that pretreatment with EA can attenuate the increased responsiveness of $[\text{Ca}^{2+}]_i$ in SGIR-subjected cardiac myocytes to the activators of AC, PKA, and L-type Ca^{2+} channel. The results indicated that the components of the β_1 -AR signaling pathway, including AC and PKA, as well as L-type Ca^{2+} channel, are involved in the mediation of the antiarrhythmic effect of EA pretreatment in the rats subjected to SGIR.

METHODS

Electro-acupuncture pretreatment. The present study was approved by the Committee on the Use of Live Animals in Research of the China Academy of Chinese Medical Sciences. Male Sprague-Dawley rats weighing 266–320 g were randomly divided into four groups, namely, normal control (NC), simulative global ischemia-reperfusion (SGIR), electro-acupuncture (EA), and NC plus EA (NCEA). Before the experiments, the animals in the EA and NCEA groups were pretreated with EA applied at bilateral Neiguan acupoints (PC6, according to the textbook of experimental acupuncture, Neiguan acupoint is located on the forelimbs) for 30 min once a day for three consecutive days. For the acupuncture manipulation, two needles, 2–3 mm apart, were inserted through the skin to a depth of about 2 mm at each Neiguan acupoint. These two needles were connected to positive and negative poles of an acupuncture apparatus. The stimulatory intensity and frequency of EA were 5 mA and 20 Hz, respectively.

Langendorff perfused isolated rat heart preparation. The Langendorff perfused isolated rat hearts were prepared for the study of arrhythmias as described previously [13]. In brief, male Sprague-Dawley rats weighing 190 to 210 g were sacrificed by decapitation with a guillotine. The hearts were removed immediately and perfused retrogradely with a Krebs-Ringer solution containing (in mM) 115 NaCl, 5 KCl, 1.2 MgSO_4 , 1.2 KH_2PO_4 , 1.25 CaCl_2 , 25 NaHCO_3 , and 11 glucose with 1% dialyzed bo-

vine serum albumin. The solution was aerated with 95% O_2 and 5% CO_2 , pH 7.4, under a pressure of 55–70 mmHg and a constant flow rate of 13 ml/min. The temperature of the hearts was maintained at 36°C. The first 10 min of perfusion allowed them to stabilize, and any heart exhibiting arrhythmia during this period was discarded. The hearts in the SGIR group or the EA group were initially perfused at a rate of 13 ml/min for 10 min, which was followed by a reduced-flow perfusion at a rate of 0.5 ml/min for 40 min. After reduced-flow perfusion, the perfusing flow was restored to the control level for 10 min. The hearts were perfused at a constant flow rate of 13 ml/min without reduced-flow perfusion for 60 min in the NC and NCEA groups. Although there were occasional arrhythmias during the low-flow period, arrhythmias were much more frequent when the flow was restored, as observed in previous studies [14] and in our preliminary study. In the present study we determined the arrhythmias in the 10 min period after the flow had been restored, namely, the reperfusion.

Measurement of ECG and the arrhythmia scoring system. ECG was continuously monitored with standard lead II throughout the experiment with a positive electrode hooked to the apex of the heart and a negative electrode at the aorta. A typical ECG trace consists of a P-wave and a QRS complex, which occurs at regular intervals. Both atrial arrhythmias, including premature atrial contraction (PAC), and ventricular arrhythmias, including premature ventricular contraction (PVC), ventricular tachycardia (VT), and ventricular fibrillation (VF), were observed within 10 min after restoration of normal perfusion in the present study. VT and VF were defined as a successive run of at least 6 PVCs of uniform and irregular QRS complex, respectively. Three or more PVCs occurring within 1 min were considered frequent. The occurrence of fewer than 3 was considered occasional.

To enable quantitative comparison, a scoring system modified from that in previous studies [15, 16] was adopted. The principles it employed were (1) ventricular arrhythmias being more severe than atrial arrhythmias; (2) the severity of ventricular arrhythmias being VF, VT, frequent PVC, and occasional PVC in descending order; (3) the longer the duration of arrhythmias or the more frequent their incidence, the greater their severity. In the present study, the score of a heart was that of the most severe type of arrhythmia the heart exhibited. The details of the scoring system are shown in Table 1.

Isolated ventricular myocytes. Ventricular myocytes of male adult Sprague-Dawley rats were isolated using a collagenase perfusion method, as described previously [17]. Immediately after a 60 min perfusion (60 min normal perfusion in the NC and NCEA groups and 10 min normal perfusion + 40 min low flow perfusion + 10 min normal reperfusion in the SGIR and EA groups), the heart was then continuously perfused at a constant flow of 10

Table 1. An arrhythmia scoring system.

Arrhythmia score	Type of arrhythmia
0	No arrhythmia
1	Atrial arrhythmias or occasional PVC
2	Frequent PVC
3	VT (1–2 episodes)
4	VT (>3 episodes) or VF (1–2 episodes)

ml/min with oxygenated Joklik-modified Eagle medium supplemented with 1.25 mM CaCl_2 and 10 mM HEPES, pH 7.4, at 37°C for 5 min, followed by another 5 min with the same solution without Ca^{2+} . Type I collagenase was added to the medium to a concentration of 125 U/ml with 0.1% bovine serum albumin. After 35–45 min of perfusion with the collagenase-containing medium, both atria were discarded. The ventricular tissues from the heart subjected to both normal perfusion and SGIR were cut into small pieces by a pair of scissors and stirred by a glass rod in the same oxygenated collagenase solution for 5 min at 37°C. The residue was filtered through a 250 μm mesh screen with 2% bovine serum albumin. More than 70% of the cells were rod shaped and not trypan blue permeable. The Ca^{2+} concentration of the Joklik solution was increased gradually to 1.25 mM in 30 min.

Loading of cells with fura-2/AM. For measurement of the electrically induced $[\text{Ca}^{2+}]_i$ transient, the cells were first loaded with fura-2/AM as a Ca^{2+} indicator, and $[\text{Ca}^{2+}]_i$ transient was determined by a spectrofluorometric method described previously [18, 19]. Isolated ventricular myocytes were incubated with fura-2/AM at a concentration of 4 μM in a Joklik solution supplemented with 1.25 mM CaCl_2 for 25 min. To remove the unincorporated dye, the cells were washed with fresh incubation solution and twice centrifuged 1 min at 80–100 g. The loaded cells were resuspended in Krebs-Henseleit buffer and maintained at room temperature (24°–26°C) for 60 min before measurement of $[\text{Ca}^{2+}]_i$ in order to allow the fura-2/AM in the cytosol to de-esterify.

Measurement of cytosolic free $[\text{Ca}^{2+}]_i$. The apparatus and fura-2 fluorescent technique have been described previously [20]. The ventricular myocytes loaded with fura-2/AM were transferred to the stage of an inverted microscope (Nikon Corp.) in a superfusion chamber at room temperature. The inverted microscope was coupled with a dual excitation spectrofluorometer (Intracellular Imaging, Inc.). The ventricular myocytes were superfused with a Krebs-Ringer solution as subscribed above with 1% dialyzed bovine serum albumin and gassed with 95% O_2 -5% CO_2 . The electrical field stimulation with 15 ms duration at 0.2 Hz produced by a stimulator was added to the myocytes. Fluorescence excitation was provided from a mercury lamp, and wavelengths were selected by computer-controlled movement of two interference filters

(340 and 380 nm) into the light path of the microscope. A wheel-installed two excitation filters rotated at a rate of 60 Hz. A dichroic mirror was used to separate the excitation and emission components in the filter container. Emission (510 nm) was collected by a photomultiplier tube (PMT). The signal from the PMT was processed by a computer and displayed digitally on the computer screen. The fluorescence ratio of 340 nm (F340) over 380 nm (F380) was used as an index of $[\text{Ca}^{2+}]_i$ because changes in the fluorescence ratio were considered to accurately reflect the fluctuations in the cytosolic Ca^{2+} of the contraction-relaxation cycle.

Drugs and chemicals. Type I collagenase, fura-2/AM, forskolin, 8-Br-cAMP, and Bay K-8644 were purchased from Sigma-Aldrich Co. All chemicals were dissolved in distilled water except fura-2/AM, Bay K-8644, and forskolin, which were dissolved in diethyl sulphoxide (DMSO). The final concentration of DMSO was less than 0.1%, which itself has no effect on $[\text{Ca}^{2+}]_i$.

Statistical analysis. All data in the present study were presented as mean $\pm$ SE. A one-way ANOVA and *t*-test were used to analyze the data in different groups. A *P* value of less than 0.05 was considered as statistical significance.

RESULTS

The effects of pretreatment with EA on cardiac arrhythmias in rats subject to SGIR

Figure 1 shows the changes in arrhythmic scores evaluated by the aforementioned scoring system in different groups. Figure 1A shows the representative tracings of ECG recorded in NC, SGIR, EA, and NCEA groups, and Fig. 1B is the statistical graphics among the groups. The arrhythmic score was zero in the NC and NCEA groups, suggesting that there was no arrhythmia. In the SGIR group there were different arrhythmias induced by myocardial ischemia and reperfusion, including atrial and ventricular arrhythmias. The arrhythmic score was 3.21 ± 0.73 in this group. With the pretreatment of EA before SGIR, the arrhythmias produced by ischemia and reperfusion were significantly blunted in the EA group, exhibiting a lower arrhythmic score of 1.24 ± 0.67 .

Effect of EA pretreatment on the forskolin-induced increase in $[\text{Ca}^{2+}]_i$ transient in single cardiac myocyte isolated from the rat hearts subjected to SGIR

Figure 2A shows the representative tracings of $[\text{Ca}^{2+}]_i$ transient in the single ventricular myocyte in response to forskolin stimulation in NC, SGIR, EA, and NCEA groups; Fig. 2B is the statistical graphics among the groups. The amplitude of electrically induced $[\text{Ca}^{2+}]_i$ transient in the single cardiac myocyte was enhanced by $77.07 \pm 3.95\%$ by forskolin, an activator of AC, at a con-

Fig. 1. Effect of EA pretreatment on cardiac arrhythmias in the isolated heart subjected to simulative global ischemia and reperfusion. **Panel A:** Representative traces of ECG showing the cardiac arrhythmias in the rats of different groups. **Panel B:** Statistical results of arrhythmic scores evaluating the cardiac arrhythmias recorded 10 min after reperfusion in the different groups. NC, normal control group; SGIR, simulative global ischemia and reperfusion group; EA, electro-acupuncture group; NCEA, normal control plus electro-acupuncture group. $**P < 0.01$ as compared with NC group; $##P < 0.05$ in comparison with SGIR group ($n = 10$ in each group above).

Fig 2. Effect of EA pretreatment on the response of $[Ca^{2+}]_i$ to forskolin in single ventricular myocyte isolated from perfused heart subjected to simulative global ischemia and reperfusion. **Panel A:** Representative traces of Ca^{2+} transient in single ventricular myocyte of different groups. **Panel B:** Statistical results of amplitude of an electrically induced $[Ca^{2+}]_i$ transient in a single ventricular myocyte of different groups. $**P < 0.01$ vs. corresponding control; $##P < 0.01$ as compared with the corresponding NC group; $++P < 0.01$ in comparison with the corresponding SGIR group. All the myocytes were stimulated by an electrical field with a duration of 15 ms and a frequency of 0.2 Hz. • Electrical stimulation ($n = 12$ in each group).

Fig. 3. Effect of EA pretreatment on the response of $[Ca^{2+}]_i$ to 8-Br-cAMP in single ventricular myocyte isolated from a perfused heart that was subjected to simulative global ischemia and reperfusion. **Panel A:** Representative traces of Ca^{2+} transient in single ventricular myocyte of different groups. **Panel B:** Statistical results of amplitude of an electrically induced $[Ca^{2+}]_i$ transient in a single ventricular myocyte of different groups. $**P < 0.01$ vs. corresponding control; $##P < 0.01$ as compared with the corresponding NC group; $+P < 0.05$, $++P < 0.01$ in comparison with the corresponding SGIR group. All the myocytes were stimulated by an electrical field with a duration of 15 ms and a frequency of 0.2 Hz. • Electrical stimulation ($n = 12$ in each group).

centration of 0.5 μ M in the NC group; The augmentation in the amplitude of $[Ca^{2+}]_i$ transient caused by forskolin in the NCEA group was $77.67 \pm 4.07\%$, almost the same as that in the NC group. In the SGIR group, the isolated hearts were subjected to simulative global ischemia followed by reperfusion. The amplitude of $[Ca^{2+}]_i$ transient was elevated by $157.87 \pm 7.89\%$ by the same concentration of forskolin in the SGIR group, which is significantly higher than that in the NC group ($P < 0.01$). As mentioned above, in the EA group the rats were pretreated with EA three times in three consecutive days before the hearts were isolated and subjected to the SGIR. It is interesting that the augmentation in the amplitude of $[Ca^{2+}]_i$ transient caused by forskolin is $78.41 \pm 4.92\%$ in the EA group, which was much lower than that in the SGIR group ($P < 0.01$) and almost the same as that in the NC group.

Effect of EA pretreatment on the 8-Br-cAMP-induced increase in $[Ca^{2+}]_i$ transient in single cardiac myocyte isolated from the rat hearts subjected to SGIR

Figure 3A shows the representative tracings of intracellular Ca^{2+} transient in the single ventricular myocyte

in response to 8-Br-cAMP stimulation in the NC, SGIR, EA, and NCEA groups; Fig. 3B shows the statistical graphics among the groups; 8-Br-cAMP, an activator of PKA, caused different augmentations in the amplitude of electrically induced $[Ca^{2+}]_i$ transient in the single cardiac myocyte. The percentage of the augmentation was $68.92 \pm 3.65\%$ in the SGIR group, which is significantly higher than $54.15 \pm 2.70\%$ in the NC group ($P < 0.01$). In the EA group, the augmentation in the amplitude of $[Ca^{2+}]_i$ transient caused by 8-Br-cAMP was $57.36 \pm 3.44\%$, which is much lower than that in the SGIR group ($P < 0.05$). The amplitude of $[Ca^{2+}]_i$ transient was elevated by $54.83 \pm 2.89\%$ by the same concentration of 8-Br-cAMP in the NCEA group.

Effect of EA pretreatment on the Bay K-8644-induced increase in $[Ca^{2+}]_i$ transient in single cardiac myocyte isolated from the rat hearts subjected to SGIR

Figure 4A shows the representative tracings of intracellular Ca^{2+} transient in the single ventricular myocyte in response to Bay K-8644 stimulation in the NC, SGIR, EA, and NCEA groups; Fig. 4B is the statistical graph-

Fig. 4. Effect of EA pretreatment on the response of $[Ca^{2+}]_i$ to Bay K-8644 in single ventricular myocyte isolated from perfused heart subjected to simulative global ischemia and reperfusion. **Panel A:** Representative traces of Ca^{2+} transient in a single ventricular myocyte of different groups. **Panel B:** Statistical results of amplitude of an electrically induced $[Ca^{2+}]_i$ transient in a single ventricular myocyte of different groups. ⁺⁺ $P < 0.01$ vs. corresponding control; ^{##} $P < 0.01$ as compared with the corresponding NC group; ⁺⁺ $P < 0.01$ in comparison with the corresponding SGIR group. All the myocytes were stimulated by an electrical field with a duration of 15 ms and a frequency of 0.2 Hz. • Electrical stimulation ($n = 12$ in each group).

ics among the groups. As shown in Fig. 4, Bay K-8644, a specific opener of L-type Ca^{2+} channel, caused different augmentations in the amplitude of electrically induced $[Ca^{2+}]_i$ transient in the above different groups. The percentage of the augmentation was $78.02 \pm 4.90\%$ in the SGIR group, which is significantly higher than $59.06 \pm 2.95\%$ in the NC group ($P < 0.01$). In the EA group, the augmentation in the amplitude of $[Ca^{2+}]_i$ transient caused by Bay K-8644 is $62.17 \pm 5.11\%$, much lower than that in the SGIR group ($P < 0.01$); and the amplitude of $[Ca^{2+}]_i$ transient was elevated $60.11 \pm 3.07\%$ by the same concentration of Bay K-8644 in the NCEA group.

DISCUSSION

During MIR, both the sympathetic activity to the heart [21–23] and the cardiac responsiveness to the sympathetic influence [23] are increased. This consequently increases $[Ca^{2+}]_i$, which has been shown to contribute to arrhythmias [24, 25]. It is well established that β_1 -AR stimulation causes the increase in $[Ca^{2+}]_i$, even $[Ca^{2+}]_i$ overload [26, 27], which may induce arrhythmias [24], via increases in cAMP production [28] and influx of Ca^{2+}

across the L-type Ca^{2+} channel [26, 29]. Further studies showed that most components of the signaling pathway of β_1 -AR were involved in the occurrence of cardiac arrhythmias. Activation of adenylate cyclase by forskolin led to arrhythmia-related cytosolic Ca^{2+} oscillation [30] and an enhancement of cytosolic cAMP production [31]. An activator of protein kinase A, 8-Br-cAMP, which phosphorylates the Ca^{2+} cycling proteins, can also cause atrial and ventricular arrhythmias [32]. Furthermore, the various cardiac arrhythmias were reported to be induced by Bay K-8644, a specific L-type Ca^{2+} channel opener [33]. It was observed in our previous study that the contents of β_1 -AR, $G_{s\alpha}$ protein, and cAMP were increased in the cardiac myocytes subjected to ischemia and reperfusion [8], and EA pretreatment could prevent the myocardium from the injury induced by ischemia and reperfusion by inhibiting the increased contents of β_1 -AR, $G_{s\alpha}$ protein, and cytosolic cAMP, the components of β_1 -AR signaling pathway. However, it is still not known whether the signaling components other than β_1 -AR, $G_{s\alpha}$ protein, and cytosolic cAMP in β_1 -AR signal transduction pathway are involved in the mediation of the antiarrhythmic effect of EA pretreatment. The aim of the present study

is to further explore the undiscovered mechanisms. The present results showed that severe arrhythmia was produced by SGIR, and the response of $[Ca^{2+}]_i$ to the activators of AC, PKA, and L-type Ca^{2+} channel in the myocytes subjected to SGIR were also enhanced, suggesting that these components of the β_1 -AR signaling pathway were involved in the mediation of myocardial injury and arrhythmias induced by myocardial ischemia and reperfusion.

It was found previously that cyclic brief ischemic preconditioning can protect the heart from injury induced by subsequent longer ischemia. Stimulating β_1 -AR with nor-epinephrine prior to ischemia can also mimic ischemic preconditioning to attenuate the ischemic myocardial injury [6, 7]. AC, PKA, and L-type Ca^{2+} channel are the important components of the β_1 -AR signaling pathway, and all of them were reported to contribute to the cardioprotection of ischemic preconditioning [34–36]. Based on these findings, we hypothesized the involvement of either AC, PKA, or L-type Ca^{2+} channel, the β_1 -AR signaling components other than β_1 -AR itself, Gs protein, and cAMP in the mediation of the antiarrhythmic effect produced by EA pretreatment. Furthermore, the intracellular calcium is well-known to be strongly modulated by β_1 -AR stimulation, and the increase or overload of intracellular calcium is associated with the arrhythmogenesis [37]. For example, the arrhythmia-related delayed afterdepolarizations can be produced by catecholaminergic stimulation, known to stimulate mainly the beta-adrenergic receptors, by increasing the intracellular calcium [38], and similarly, early afterdepolarizations can also be induced by an LCC opener such as Bay K-8644 [39]. It was also demonstrated that acupuncture at the acupoints of Stomach Meridian of Foot-Yangming enhanced significantly the intracellular calcium in gastric smooth muscle cells. All the findings above suggest that acupuncture may produce the antiarrhythmic effect via influencing the arrhythmogenic $[Ca^{2+}]_i$ or its modulators, including β_1 -AR signaling components. Thus we determined the response of $[Ca^{2+}]_i$ in cardiac myocytes subjected to SGIR to the activators of AC and PKA and the opener of L-type Ca^{2+} channel, the important components of the β_1 -AR signaling pathway. As we supposed, EA pretreatment can produce antiarrhythmic effect in the rat of SGIR, which was supported by the data showing that the SGIR-induced augment of arrhythmia score was significantly attenuated after repetitive EA pretreatment. Of greater interest, the further experiments showed that pretreatment with EA also attenuated the SGIR-increased response of $[Ca^{2+}]_i$ to the activators of AC, PKA, and the L-type Ca^{2+} channel in single cardiomyocytes, suggesting that AC, PKA, and L-type Ca^{2+} channel are involved in the mediation of the antiarrhythmic effect of the acupuncture pretreatment. Moreover, EA stimulation was shown to significantly enhance the sympathetic activity [40]. Theoretically,

the augmented sympathetic activity can consequently increase the release of catecholamine that stimulates cardiac β_1 -AR and finally enhances the $[Ca^{2+}]_i$. As mentioned above, there was a study showing that acupuncture at the acupoints of Stomach Meridian of Foot Yangming enhanced the resting $[Ca^{2+}]_i$ in the smooth muscle cells isolated from stomachs of the normal rabbits. Strangely, our present data showed that EA pretreatment did not significantly influence the $[Ca^{2+}]_i$ transient in the single cardiomyocyte isolated from the normal rats in the NCEA group. A reasonable explanation for the unchanged $[Ca^{2+}]_i$ following the EA pretreatment in the normal rats is that although EA can enhance the $[Ca^{2+}]_i$ momentarily during the stimulation by exciting the sympathetic activity to stimulate the cardiac β_1 -AR, the enhanced $[Ca^{2+}]_i$ may restore to the normal level after the procedures of cell isolation from heart and fura-2/AM loading to the cells. However, in the present study the enhancement of $[Ca^{2+}]_i$ induced by ischemia and reperfusion was not restored to the normal level even after the same procedures of cell isolation and fura-2 loading in the SGIR group, which is in agreement with the recent study on the role played by $[Ca^{2+}]_i$ in the MIR. The results suggest that the impairment of the mechanisms of handling $[Ca^{2+}]_i$ may be too severe to be recovered in the period. As described in Methods of the present study, the rats were pretreated with EA three times in three consecutive days before the cardiomyocytes were isolated from the perfused heart subjected to SGIR. The EA pretreatment-induced repetitive enhancement-restoration cycles of $[Ca^{2+}]_i$ via stimulating cardiac β_1 -AR may mimic the repetitive ischemic preconditioning to produce the cardioprotective effect in the rats subjected to SGIR. The underlying mechanisms for the cardioprotection produced by EA pretreatment may be to induce the possible adaptation (or desensitization) of the cardiomyocytes isolated from the heart with SGIR to the stimulation of β_1 -AR and its signaling components, which are overactivated during ischemia and reperfusion.

CONCLUSION

The results indicate that the repetitive EA pretreatment can produce antiarrhythmic effect by inhibiting the function of AC, PKA, and the L-type Ca^{2+} channel.

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