Effect of genistein on L-type calcium current in guinea pig ventricular myocytes

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Abstract: This paper was aimed to study the effect of genistein (GST) on L-type calcium current (I_{Ca,L}) in isolated guinea pig ventricular myocytes using whole cell patch-clamp recording technique. The results are as follows. (1) GST (10, 50, 100 µmol/L) reduced the voltage-activated peak amplitude of I_{Ca,L} in a concentration-dependent manner. Daidzein (100 µmol/L), a structural analogue of GST which has little or no inhibitory effect on tyrosine kinase, produced no effect over the same concentration range on I_{Ca,L} (n=5, P>0.05). (2) GST up-shifted the current-voltage (I-V) curve, but the characteristics of I-V relationship were not significantly altered, and the maximal activation voltage of I_{Ca,L} was not different from that of control. GST did not affect the activation kinetics of I_{Ca,L}. (3) GST markedly shifted the steady-state inactivation curve of I_{Ca,L} to the left, and accelerated the voltage-dependent steady-state inactivation of I_{Ca,L}. V_{0.5} value was −28.6 ± 0.6 mV in the control and −32.8 ± 1.1 mV in the presence of GST. The κ values were 5.8 ± 0.5 mV and 6.5 ± 0.9 mV, respectively (n=6, P<0.05). (4) GST markedly shifted the curve of time-dependent recovery of I_{Ca,L} from the steady-state inactivation to the right, and slowed down the recovery of I_{Ca,L} from inactivation (n=7, P<0.01). (5) Sodium orthovanadate (1 mmol/L), a potent inhibitor of tyrosine phosphatase, significantly inhibited GST-induced inhibition (n=6, P<0.01). From the results obtained it is concluded that genistein inhibits I_{Ca,L} and acts on the inactivated state of L-type calcium channel. This inhibitory effect of GST involves protein tyrosine kinase inhibition in guinea pig ventricular myocytes.

Key words: genistein; patch-clamp techniques; myocardium; L-type calcium channels

三羟异黄酮对豚鼠心室肌细胞 L-型钙通道电流的影响

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摘 要: 本实验用单细胞膜片钳技术观察三羟异黄酮 (genistein, GST) 对豚鼠心室肌细胞 L- 型钙通道电流 (I_{Ca,L}) 的影响。结果如下: (1) GST (10, 50, 100 µmol/L) 可浓度依赖性地降低 I_{Ca,L} (n=6, P<0.01)。GST 的非活性结构类似物 daidzein (100 µmol/L) 在同一浓度范围对 I_{Ca,L} 没有影响 (n=5, P>0.05)。 (2) GST 使 I-V 曲线上移, 但对 I_{Ca,L} 的电压依赖特性和最大激活电压无明显影响。 (3) GST 对 I_{Ca,L} 的激活动力学特性也无影响。但可使电流稳态失活曲线左移。V_{0.5} 从对照的 −28.6 ± 0.6 mV 变为 −32.8 ± 1.1 mV, κ 值从对照的 5.8 ± 0.5 mV 升至 6.5 ± 0.9 mV (n=6, P<0.05)。 (4) GST 明显使复合线右移, 从而使 I_{Ca,L} 从失活状态下恢复明显减小 (n=7, P<0.01)。 (5) 钴酸钠磷酸酯抑制剂亚砷酸钠 (1 mmol/L) 显著对抗 GST 引起的 I_{Ca,L} 抑制效能 (n=6, P<0.01)。根据以上结果得出的结论是: GST 抑制 I_{Ca,L}, 加速钙通道失活和钙通道在失活状态下恢复减慢; GST 对 I_{Ca,L} 的这种抑制作用与蛋白酪氨酸磷酸酶 (PTK) 抑制有关。

关键词: 三羟异黄酮；膜片钳技术；心肌；L- 型钙通道

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Phytoestrogens are plant-derived diphenolic compounds, which are structurally and functionally similar to estradiol. A growing number of reports have documented that phytoestrogens may confer cardiovascular protection [1-3]. Genistein (GST), one of the most well-known phytoestrogens, is an isoflavone that is proved to be a specific inhibitor of protein tyrosine kinase (PTK) [4]. Our previous works demonstrated that GST reduced the amplitude of action potential (APD), maximal rate of depolarization (V\textsubscript{m}) overshoot (OS), velocity of diastolic (phase 4) depolarization (VDD) and rate of pacemaker firing (RPF) in sinoatrial node pacemaker cells of rabbits, and human atrial fibers [5,6]. Moreover, GST inhibited early afterdepolarization (EDA) and activity triggered by ouabain in guinea pig papillary muscles [7]. Our recent study indicated that GST had protective effects against myocardial ischemia-reperfusion injury in rabbits [8] and may exert its action by affecting the calcium influx [9]. It was reported that GST inhibited L-type calcium current by a PTK-dependent mechanism [10]. However, contradictory results had been demonstrated that this effect induced by GST was PTK independent [11]. The exact mechanism responsible for the discrepancy is not clear. The present study was undertaken to observe the effect of GST on L-type calcium current and define its mechanism in guinea pig ventricular myocytes using whole cell patch-clamp recording technique.

1 MATERIALS AND METHODS

1.1 Isolation of ventricular myocytes. Guinea-pig ventricular myocytes were obtained by modified enzymatic dissociation technique [12]. Male guinea pigs weighing 280–340 g were provided by Experimental Animal Center of Hebei Province (grade II, Certificate No. 04064). The guinea pigs were stunned by heavy blow on the head. The hearts with the aorta of 2–3 mm length were removed and placed in oxygenated ice-cold Ca\textsuperscript{2+}-free Tyrode’s solution. A Langendorff retrograde perfusion was performed through the aorta at a rate of 9 ml/min with Ca\textsuperscript{2+}-free Tyrode’s solution for 5 min and then with the same solution containing 34 μmol/L CaCl\textsubscript{2} and 360 mg/L collagenase (Sigma, type II) for 9 min at 37°C. The ventricles were incubated in Ca\textsuperscript{2+}-free Tyrode’s solution at room temperature for about 30 min. Afterwards, a piece of ventricle was cut off and teased into smaller pieces in KB solution. Myocytes were harvested after filtration through a 200 μm nylon mesh and stored in KB solution for at least 1 h before the experiment. The concentration of Ca\textsuperscript{2+} in Tyrode’s solution was gradually increased to 1.0 mmol/L. All experiments were performed within 12 h after isolation.

1.2 Measurement of Ca\textsuperscript{2+} current. Isolated ventricular myocytes were placed in the experimental chamber (0.4 ml) mounted on the stage of an inverted microscope (CK2, Olympus). After setting to the bottom of chamber, the cells were superfused with external solution for 10 min at a rate of 2–3 ml/min at 25°C. Transmembrane currents were recorded with an Axopatch amplifier (200B, Axon Instruments, USA). Glass microelectrodes were made using a microelectrode puller (PB-7, Narishige, Japan) by two-stage pulling and had a resistance of 2.0 to 4.0 MΩ, when filled with electrode internal solution. Only the rod shaped cells with visible striations were used for experiments. Liquid junction potential between the pipette solution and external solution was corrected after the pipette tipped into the external solution. After gigaseal formation, the membrane was ruptured with a gentle suction to obtain the whole cell voltage-clamp configuration. To minimize the duration of capacitive current, membrane capacitance and series resistance were compensated after membrane rupture. The external solution was changed to Na\textsuperscript{+}-free solution in which Na\textsuperscript{+} was replaced by equimolar tetraethylammonium chloride (TEA-Cl). Na\textsuperscript{+} current was also inactivated at the holding potential (E\textsubscript{h}) of −40 mV and blocked by tetrodotoxin (TTX, 2×10\textsuperscript{−6} mol/L). K\textsuperscript{+} current was suppressed by substituting intracellular K\textsuperscript{+} by Cs\textsuperscript{+}. Computer-generated voltage or current pulses were programmed using the pCLAMP 6.0 software (Axon Instruments, USA). On-line acquired data were stored on a hard disk of the microcomputer. All experiments were carried out at room temperature (22–24°C).

1.3 Solutions and drugs. Genistein, collagenase type II, bovine serum albumin (BSA), taurine, TEA-Cl, HEPES, egtazic acid, Cs\textsubscript{2}OH, Cs\textsubscript{2}Cl, and MgATP were purchased from Sigma Co. Tetrodotoxin (TTX) was purchased from Hebei Ocean Product Institute of China. The Ca\textsuperscript{2+}-free Tyrode’s solution contained NaCl 100, KCl 10, MgSO\textsubscript{4} 5.0, NaH\textsubscript{2}PO\textsubscript{4} 1.2, glucose 20, taurine 10, and MOPS 10 mmol/L, and the pH was adjusted to 7.4 with NaOH. The electrode internal solution for whole-cell recording was composed of MgATP 3, CsCl 140, HEPES 10, egtazic acid 10 mmol/L, pH was adjusted to 7.2 with CsOH. The external solution was composed of TEA-Cl 140, MgCl\textsubscript{2} 2.0, CaCl\textsubscript{2} 1.5, glucose 10, HEPES 10, and TTX 0.002 mmol/L, gassed with 100% O\textsubscript{2}, and the pH was adjusted with TEAOH to 7.3–7.4. GST was dissolved in DMSO and diluted in external solution at the concentra-
tions of 10, 50, and 100 µmol/L before experiment.

1.4 Statistics. The values were expressed as means ± SD. Statistical analysis was performed using t-test and P<0.05 was defined as significant.

2 RESULTS

2.1 Effect of GST and daidzei on L-type calcium current

L-type Ca²⁺ current in guinea pig ventricular myocytes was evoked by a depolarizing step pulse from the holding potential (E_h) of –40 mV to 0 mV at the frequency of 0.1 Hz. The step pulse duration was 350 ms. GST (10, 50 and 100 µmol/L) inhibited the peak amplitude of I_{ca,L} in a concentration-dependent manner (Fig. 1A). The inhibitory effect of GST remained even after 5- min washout with control solution. Daidzein (100 µmol/L), a structural analogue of GST which has little inhibitory effect on tyrosine kinase activity, had no effect on I_{ca,L} (data not shown).

2.2 Effect of GST on current-voltage relationship of I_{ca,L}

Current-voltage (I-V) curve of L-type Ca²⁺ current was obtained by a number of depolarizing step pulses (350 ms) from the E_h of –40 mV to test potentials between –50 mV and 50 mV. The test step pulses were delivered in 10 mV increments. I_{ca,L} was activated at –30 mV and the peak amplitude occurred at the potential of 0 mV. GST (10, 50 and 100 µmol/L) upshifted the I-V curve, but the current density at test potential of 0 mV was decreased from –12.6 ± 1.5 pA/pF to –11.4 ± 2.3 pA/pF, to –10.5 ± 0.8 pA/pF and to –5.4 ± 2.1 pA/pF (n=6 cells from 5 hearts, P<0.01), respectively (Fig. 1B).

2.3 Activation and inactivation kinetics of I_{ca,L}

To investigate the effects of GST on the voltage dependence of activation and inactivation, steady-state activation and inactivation curves were obtained before and after GST application. The activation curves were derived from the current/voltage relationships (Fig. 2). The activation curves were fitted according to the Boltzmann equation: \( I/I_{\text{max}} = 1/[1+\exp[(V-V_{0.5})/\kappa]] \). The half activation potential (V_{0.5}) and the slope factor (κ) were –6.9± 1.2 mV and 4.7±1.0 mV in control, and –4.9 ± 1.8 mV and 5.3 ± 1.5 mV after GST (100 µmol/L) application. These values were not significantly different between the groups (n=6 cells from 5 hearts, P>0.05). Steady-state inactivation of the L-type channel was obtained by a double-pulse protocol[13]. Membrane potential is first stepped from –60 mV to 30 mV for 1000 ms and then to +10 mV for 300 ms (test pulse) and finally clamped back to the holding potential of –60 mV at the pulse frequency of 0.1 Hz. The peak current elicited by test pulses was normalized by the maximum current and plotted against the conditioning potential.

Fig. 1. Effect of GST on I_{ca,L} in isolated guinea pig ventricular myocytes. A: Currents were recorded during 350 ms depolarization from the holding potential of –40 mV to 0 mV. B: Effect of GST on I-V curve of I_{ca,L} in isolated guinea pig ventricular myocytes (n=6 cells from 5 hearts).

Fig. 2. Effects of GST on steady-state activation and inactivation kinetics of I_{ca,L} in myocytes.
The inactivation curves were also fitted according to the Boltzmann equation. GST (100 µmol/L) shifted the $V_{0.5}$ from $-28.6 \pm 0.6$ mV to $-32.8 \pm 1.1$ mV and shifted $\kappa$ from $5.8 \pm 0.5$ mV to $6.5 \pm 0.9$ mV ($P<0.05$). However, the slope factor showed no change ($P>0.05$) ($n=6$ cells from 5 hearts) (Fig. 2).

### 2.4 Effect of GST on recovery of $I_{\text{caL}}$ from inactivation

The recovery of $I_{\text{caL}}$ from inactivation was studied using double-pulse protocol consisting of a 300 ms repulse to $+10$ mV (P1) followed by a 300 ms test pulse to $+10$ mV (P2) after an interval between 0 and 650 ms at the holding potential of $-60$ mV. Double-pulse stimulation was repeated every 6 s. GST (100 µmol/L) markedly shifted the recovery curve of $I_{\text{caL}}$ to the right, indicating slower recovery of $I_{\text{caL}}$ from inactivation ($n=7$ cells from 6 hearts, $P<0.01$) (Fig. 3).

### 2.5 Effects of sodium orthovanadate on GST-induced $I_{\text{caL}}$ change

To further assess the mechanism underlying the inhibitory effect of GST on $I_{\text{caL}}$, we observed the effect of sodium orthovanadate (1 mmol/L), a potent inhibitor of protein tyrosine phospatase, on the inhibitory effect of GST. Pretreatment with sodium orthovanadate (1 mmol/L), the GST-induced reduction in $I_{\text{caL}}$ was significantly attenuated ($n=6$ cells from 6 hearts, $P<0.01$) (Fig. 4).

### 3 DISCUSSION

In this study, we found that GST concentration-dependently decreased $I_{\text{caL}}$ and shifted upward the $I-V$ curve, but the maximal activation of $I_{\text{caL}}$ was not changed. Such effects indicated that GST could block L-type calcium channel in guinea pig ventricular myocytes, and had no marked effect on characteristics of voltage-dependence and activation kinetics of $I_{\text{caL}}$. The inactivation kinetics of $I_{\text{caL}}$ were changed by GST. It shifted steady-state inactivation curve to the left, and accelerated the voltage-dependent steady-state inactivation of the calcium channel. In addition, GST (100 µmol/L) markedly shifted the recovery curve of $I_{\text{caL}}$ to the right. Therefore, these findings indicate that the myocardial electrophysiological effects of GST observed in our previous studies[5-8] might be attributed to its action on $I_{\text{caL}}$.

Tyrosine kinase activation is thought to contribute to cell survival, proliferation, and differentiation in many cell types[14]. Furthermore, several lines of evidence showed[15,16] that phosphorylation by tyrosine kinase modulated ion channel activity. In this study, we showed that GST, a specific inhibitor of PTK, decreased the $I_{\text{caL}}$ in the ventricular myocytes. After pretreatment with sodium orthovanadate (1 mmol/L), a potent inhibitor of tyrosine phosphatase, the GST-induced reduction in $I_{\text{caL}}$ was markedly antagonized, suggesting that tyrosine kinase activity can directly regulate cardiac L-type Ca$^{2+}$ channels. Nevertheless, contradictory results have been shown that the effect induced by GST was not related to the suppression of PTK activity but to direct effects of these drugs on L-type Ca$^{2+}$ channels[17,18]. Belevych et al.[19] also reported that GST inhibits cardiac L-type Ca$^{2+}$ channels activity by a tyrosine kinase-independent mechanism. The exact mechanism for this discrepancy remains to be established. Therefore, in addition to tyrosine kinase inhibition, the effects of genistein on cardiovascular system may be much more complicated than we expected.

To further assess the mechanism underlying the inhibi-
tory effect of GST on $I_{\text{Ca,L}}$, we observed the action of daidzein (100 µmol/L), a structural analogue of GST with a little inhibitory effect on tyrosine kinase activity[4]. It had no effect on $I_{\text{Ca,L}}$ (data not shown). This result was consistent with the findings by other workers[11,19].

In conclusion, GST inhibited $I_{\text{Ca,L}}$ in guinea pig ventricular myocytes. This effect may be caused by accelerating the inactivation of calcium channel, and slowing down the recovery of calcium channel from inactivation via tyrosine kinase inhibition.

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