

BASIC RESEARCH

Effect of genistein on voltage-gated potassium channels in guinea pig proximal colon smooth muscle cells

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Abstract

AIM: To investigate the action of genistein (GST), a broad spectrum tyrosine kinase inhibitor, on voltage-gated potassium channels in guinea pig proximal colon smooth muscle cells.

METHODS: Smooth muscle cells in guinea pig proximal colon were enzymatically isolated. Nystatin-perforated whole cell patch clamp technique was used to record potassium currents including fast transient outward current (I_{Kto}) and delayed rectifier current (I_{Kdr}), two of which were isolated pharmacologically with 10 mmol/L tetraethylammonium or 5 mmol/L 4-aminopyridine. Contamination of calcium-dependent potassium currents was minimized with no calcium and 0.2 mmol/L $CdCl_2$ in an external solution.

RESULTS: GST (10-100 μ mol/L) reversibly and dose-dependently reduced the peak amplitude of I_{Kto} with an IC_{50} value of 22.0 ± 6.9 μ mol/L. To a lesser extent, I_{Kdr} was also inhibited in both peak current and sustained current. GST could not totally block the outward potassium current as a fraction of the outward potassium current, which was insensitive to GST. GST had no effect on the steady-state activation ($n=6$) and inactivation kinetics ($n=6$) of I_{Kto} . Sodium orthovanadate (1 mmol/L), a potent inhibitor of tyrosine phosphatase, significantly inhibited GST-induced inhibition ($P < 0.05$).

CONCLUSION: GST can dose-dependently and reversibly block voltage-gated potassium channels in guinea pig proximal colon smooth muscle cells.

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Key words: Protein tyrosine kinase; Patch-clamp technique; Genistein; Voltage-gated potassium channel; Colon; Smooth muscle cells

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INTRODUCTION

Ion channels are targets of many intracellular signaling pathways, including protein phosphorylation and dephosphorylation. These processes can modify channel activity and dramatically alter the electrophysiological properties of both excitable and nonexcitable cells^[1]. In addition to the extensive information available about the regulation of ion channels by serine-threonine kinases, an emerging body of evidence suggests that channels are also regulated by phosphorylation on tyrosine residues, such as ligand-gated channels AChR^[2], NMDA receptor^[3], potassium channels^[4,5] as well as calcium channels^[6,7]. GST, a specific inhibitor of tyrosine kinases, inhibits visceral smooth muscle contraction induced by tyrosine phosphatase inhibitor vanadate^[8], angiotensin II^[9] and carbachol^[10] via tyrosine kinase-mediated process. Potassium channels, which are substrates for protein phosphorylation and dephosphorylation, control the contraction of gastrointestinal smooth muscles by setting resting potential and influencing slow waves and action potential configuration^[11]. However, little is known of the modulation of potassium channels in gastrointestinal smooth muscle cells via tyrosine kinase pathway. This study was to investigate the effects and mechanisms of GST on voltage-gated potassium channels (Kv) in smooth muscle cells of guinea pig proximal colon.

MATERIALS AND METHODS

Cell dissociation

Smooth muscle cells were enzymatically isolated using modified procedures as previously described^[12]. Briefly, male guinea pigs (200-350 g) were killed by cervical dislocation and proximal colon about 2 cm aboral to cecum was rapidly excised. Under anatomical microscope, smooth muscle strips were dissected out, cut into small pieces and incubated for 30 min in low calcium solution containing 10 mmol/L HEPES, 135 mmol/L NaCl, 6 mmol/L KCl, 0.05 mmol/L $CaCl_2$, 1.2 mmol/L $MgCl_2$, 10 mmol/L Glucose, pH 7.4. The pieces of muscles were then transferred to low calcium solution containing

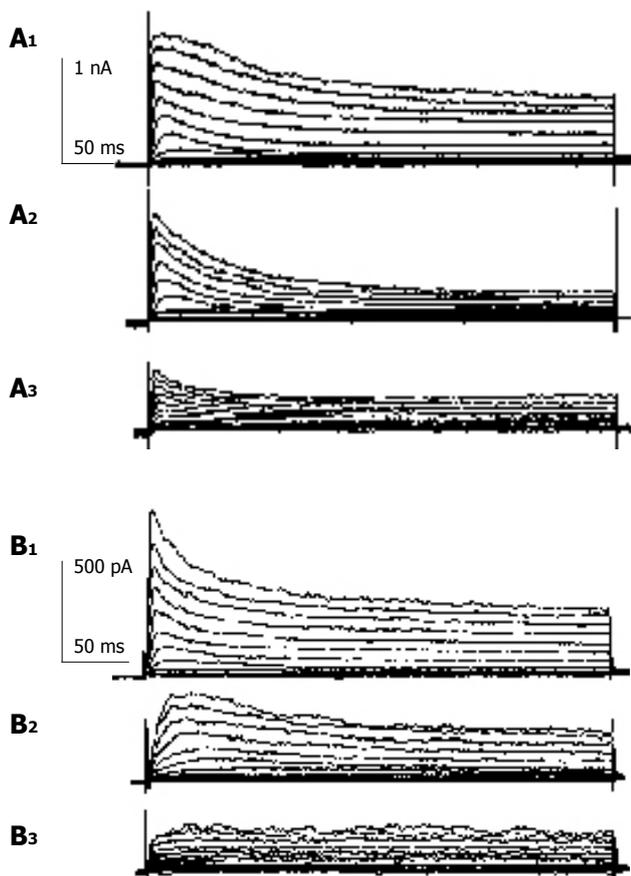


Figure 1 Two components of voltage-gated potassium currents in guinea pig colon smooth muscle cells and effect of GST. **A₁-A₃**: Currents recorded every 10 s between -80 and +50 mV then back to -40 mV with holding potential -80 mV in the control (**A₁**), in the presence of 10 mmol/L TEA (**A₂**) and TEA and 30 μmol/L GST (**A₃**). **B**: Currents recorded every 10 s between -80 and +50 mV with holding potential -80 mV in the control; **B₁**: current in the presence of 5 mmol/L 4-AP developed slowly and showed slower inactivation over 400 ms; **B₃**: effect of 50 μmol/L GST in the presence of 5 mmol/L 4-AP.

3 g/L papain, 2 g/L DTT, 2 g/L bovine serum albumin. Tissues were incubated at 36 °C in enzyme solution for 15 min and then suspended in enzyme-free low calcium solution. Tissue pieces were gently agitated to create a cell suspension. Dispersed cells were stored at 4 °C for later use. Experiments were performed at 20-22 °C within 10 h.

Perforated whole cell voltage clamp recording

Relaxed single colon smooth muscle cells with smooth appearance and spindle shape observed under an inverted microscope (IX70, Olympus) were used. Myocytes were perfused with Ca²⁺-free cell bath solution in a self-made small volume chamber in which solution could be exchanged in 30 s. The composition of bath solution was the same as that of low calcium solution except for exclusion of calcium and inclusion of 0.2 mmol/L CdCl₂ to minimize the contamination of calcium-dependent potassium currents.

Patch clamp micropipettes were pulled with a programmable puller (P-97, Sutter Instruments) and their tips were fire polished (CPM-2, ALA Co.). The pipette resistance was 3-5 MΩ. Currents were amplified with Axopatch 200B (Axon Instruments). Data were filtered at 1 kHz and analyzed with pClamp software (version 8.2).

Nystatin-perforated whole cell patch clamp technique was used to record voltage-gated potassium currents. Nystatin was dissolved in DMSO at a concentration of 25 g/L, and then added to the internal pipette solution to yield a final nystatin concentration of 100 mg/L. The internal solution contained 10 mmol/L HEPES, 110 mmol/L glucoante (potassium salt), 30 mmol/L KCl, 10 mmol/L NaCl, 2 mmol/L MgCl₂, pH 7.2. After giga seals were obtained, access resistance was monitored for 10 min to allow the drop of access resistance (average 17.7 ± 5.7 MΩ, $n=28$) and then compensated at 70%. Macroscopic current values were normalized for cell capacitance as whole cell current densities (pA/pF). The average cell capacitance was 41.3 ± 7.5 pF ($n=28$ cells).

Drugs and chemicals

Papain, 4-aminopyridine (4-AP), nystatin, tetraethylammonium (TEA), sodium orthovanadate (VAN), genistein (GST) were purchased from Sigma. GST was prepared as a 50 mmol/L stock solution in DMSO and stored at -20 °C. DTT (BBI), HEPES, DMSO, bovine serum albumin were from Shanghai Sangon Biological Engineering Technology and Services Company.

Statistical analysis

Data were expressed as mean ± SD. Differences in the data were evaluated by paired or independent *t*-test when appropriate. $P < 0.05$ was considered statistically significant. Software Microcal Origin 5.0 was used for statistical analysis and graph plotting.

RESULTS

Potassium currents in single smooth muscle cells

Outward potassium currents are mainly composed of voltage-gated potassium current and calcium-dependent potassium current. Under the conditions of our recordings (no added Ca²⁺ and 0.2 mmol/L CdCl₂ in the bath solution), contamination of currents through calcium-dependent potassium currents was minimized.

Currents were evoked using standard stimulus protocol, i.e., the membrane potential was stepped for 400 ms from a holding potential of -80 mV to test potentials between -80 and +50 mV in 10 mV increments. Depolarization to potentials positive to -40 mV activated non-linear, time-dependent outward currents which could be divided into transient outward potassium current (I_{kto}) and delayed rectifier potassium current (I_{kdr}) as shown in Figure 1. K_{to} was sensitive to millimolar concentration of 4-AP and insensitive to TEA. K_{dr} was on the contrary. When 10 mmol/L TEA was externally applied, early peak current of transient outward current (peak current among the first 50 ms of test pulse, I_{peak}) was only slightly decreased, but the quasi steady state current (average current from 350 to 400 ms after test pulse onset, I_{ss}) was blocked -60% (Figure 1A). When 5 mmol/L 4-AP was externally applied, I_{peak} was much reduced (Figure 1B) and the time to half-maximum current at test potential was significantly increased (data not shown), I_{ss} was little affected. Detailed description of voltage-dependent potassium current of guinea pig proximal colon smooth muscle cells could

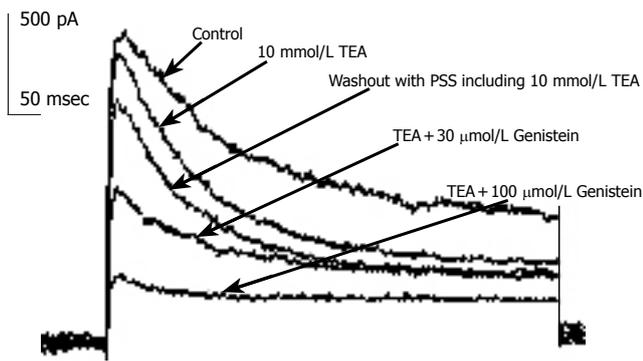


Figure 2 Concentration-dependent inhibition of transient outward potassium currents by genistein in guinea pig colon smooth muscle cells. Currents were elicited by depolarization to +50 mV for 400 ms then back to -40 mV from holding potential of -80 mV. Washout was performed for about 2 min.

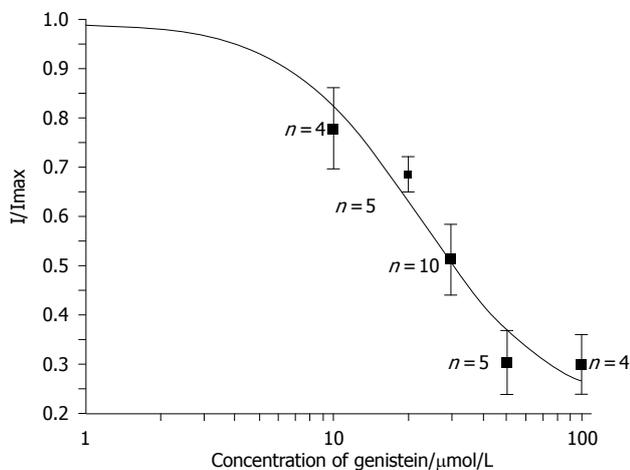


Figure 3 Average concentration-dependent transient outward potassium currents inhibited by genistein. Peak currents were measured and normalized by control current amplitude. The smooth line represents the best fitting with Hill equation. Data are expressed as mean±SE. The number of cells used in each concentration is indicated besides the error bars.

be seen elsewhere^[13], which agrees well with our study. According to the different sensitivity to TEA and 4-AP, we isolated these two kinds of current pharmacologically to study the effect of GST independently.

Concentration-dependent inhibition of GST on I_{Kto}

Currents recorded were mainly I_{Kto} when 10 mmol/L TEA was externally applied to bath solution. Repetitive single current traces were elicited for 400 ms from a holding potential of -80 mV to test potentials +50 mV in every 20s until stable currents were recorded. I_{peak} of K_{to} decreased progressively and reached a stable state in about 1-2 min after the external perfusion of GST. GST (10-100 μ mol/L) induced a reduction in I_{peak} , with little effect on the steady state potassium current. The inhibitory effects of GST were reversible (Figure 2). Perfusion of calcium-free saline including TEA could restore the amplitude of currents to about 80% of control in 2 min (data not shown).

GST could not totally block the outward potassium currents even when 100 μ mol/L GST was applied. Since no significant difference in the inhibitory effect was

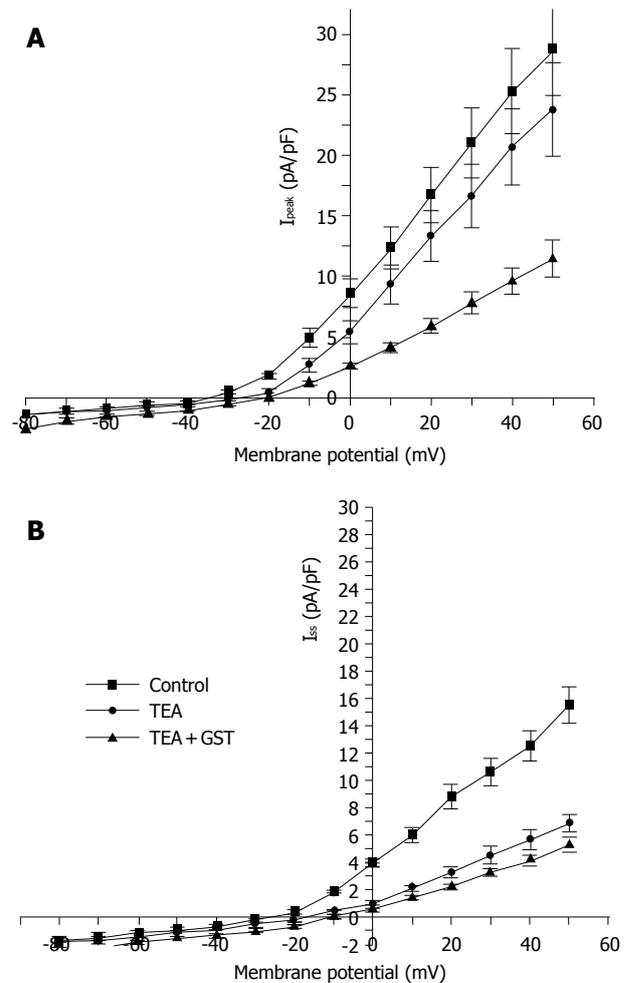


Figure 4 Effect of genistein (30 μ mol/L) on current-voltage relationship of transient outward potassium current (I_{Kto}) in guinea pig proximal colon smooth muscle cells in the presence of TEA (10 mmol/L) and Cd^{2+} (0.2 mmol/L). **A:** I - V curves of peak current density of voltage-gated potassium channels under control condition (solid square), in the presence of 10 mmol/L TEA (solid circle) as well as 10 mmol/L TEA and 30 μ mol/L genistein (solid triangle); **B:** I - V curves of steady state current density of voltage-gated potassium channels under control condition, in the presence of 10 mmol/L TEA as well as 10 mmol/L TEA and genistein (30 μ mol/L). $n=6$ cells.

found between 50 ($n=10$) and 100 ($n=5$) μ mol/L GST, a fraction of the outward potassium current was insensitive to GST.

Early peak transient outward potassium current of K_{to} measured at +50 mV test pulse from a holding potential of -80 mV was used as an index of inhibition, which was plotted as a function of GST concentration (10-100 μ mol/L). The data were fitted with $I_{GST}/I_{control} = 1 / \{1 + (IC_{50}/[D])^n\}$, where $[D]$ is the concentration of GST used, IC_{50} is the concentration at half maximal inhibition, and n is the Hill coefficient. Figure 3 shows that the effect of GST on I_{peak} of K_{to} was concentration-dependent, with an IC_{50} of 22.0 ± 6.9 μ mol/L.

Effect of GST on current-voltage relationship (I - V curves) of I_{Kto}

Currents were evoked using standard stimulus protocol. The membrane potential was stepped for 400 ms from a holding potential of -80 mV to test potentials between -80

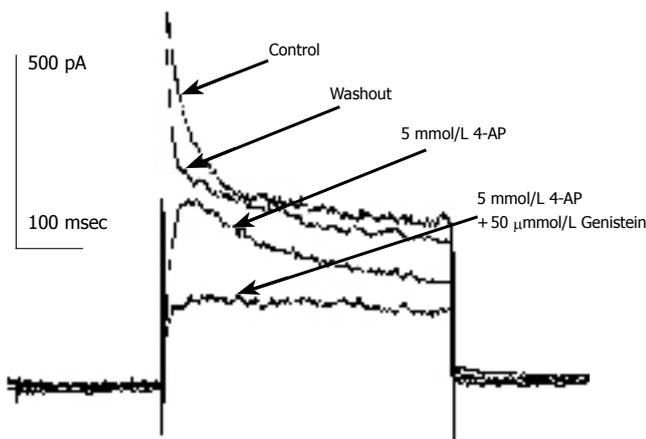


Figure 5 Effect of genistein on delayed rectifier potassium currents in the presence of 4-AP in guinea pig colon smooth muscle cells. Currents were elicited by depolarization to +50 mV for 400 ms then back to -40 mV from holding potential of -80 mV. Washout was performed for about 2 min.

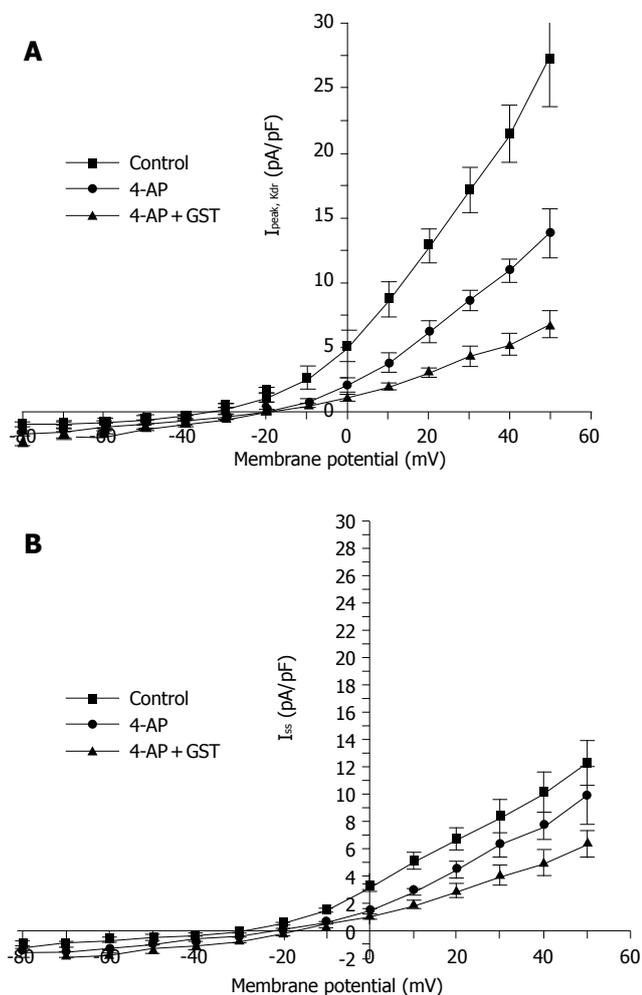


Figure 6 Effect of genistein (50 $\mu\text{mol/L}$) on current-voltage relationship of delayed rectifier potassium current (I_{Kdr}) in guinea pig proximal colon smooth muscle cells in the presence of 4-AP (5 mmol/L) and Cd^{2+} (0.2 mmol/L). **A:** I - V curves of peak current density of voltage-gated potassium channels under control condition, in the presence of 5 mmol/L 4-AP as well as 5 mmol/L 4-AP and 50 $\mu\text{mol/L}$ genistein; **B:** I - V curves of steady state current density of voltage-gated potassium channels under control condition, in the presence of 5 mmol/L 4-AP as well as 5 mmol/L 4-AP and 50 $\mu\text{mol/L}$ genistein. $n=4$ cells.

and +50 mV in 10 mV increments. The interval between two pulses was set at 10 s to allow inactivated conductance to recover. I_{peak} and I_{ss} were measured at test pulses from -80 to +50 mV and converted into current densities, which were plotted as a function of test pulse.

Figure 1 shows that in the presence of 10 mmol/L TEA, 30 $\mu\text{mol/L}$ GST significantly blocked I_{peak} ($P<0.05$, $n=6$ cells) and only slightly reduced I_{ss} . Figure 4 shows the averaged I - V curves of I_{Kto} inhibited by GST. The inhibition showed no voltage dependence.

Effect of GST on I_{Kdr}

Currents recorded in the presence of external 5 mmol/L 4-AP were mainly delayed rectifier potassium currents. Repetitive single current traces were elicited for 400 ms from a holding potential of -80 mV to test potentials +50 mV in every 20 s until stable currents were recorded. In the presence of 5 mmol/L 4-AP, 50 $\mu\text{mol/L}$ GST reduced peak current and steady state current with a fractional inhibition of current f [$f=(1-I_{\text{GST}}/I_{\text{drug}})\times 100\%$] of $52\pm 8\%$ ($P<0.05$, $n=4$) and $33\pm 2\%$ ($P<0.05$, $n=4$), respectively. The inhibitory effects of GST were reversible. Perfusion of calcium-free saline could restore the amplitude of currents in 2 min (data not shown). Details are shown in Figure 5.

Effect of GST on I - V curves of I_{Kdr}

The same experimental protocol as that of the study on the effect of GST on I_{Kto} was used. Figure 1B shows that in the presence of 5 mmol/L 4-AP, 50 $\mu\text{mol/L}$ GST significantly blocked I_{peak} as well as I_{ss} ($P<0.05$, $n=4$ cells). Figure 6 shows the averaged I - V curves of I_{Kdr} inhibited by GST. The inhibition showed no voltage dependence, and 50 $\mu\text{mol/L}$ GST had a greater effect on I_{Kto} than on I_{Kdr} ($P<0.05$).

Effect of GST on steady state activation kinetics of I_{Kto}

The activation curves of I_{Kto} were derived from I - V curves of I_{Kto} . G_{Kto} was calculated by dividing the initial peak current value by their driving force and plotted as a function of membrane potential. The activation curves were fitted with a Boltzmann function $G/G_{\text{max}}=1/\{1+\exp[(V-V_h)/\epsilon]\}$, where V is membrane potential, V_h is the half-maximal activation voltage, and ϵ is the slope constant (mV). GST had no significant effect on steady state activation kinetics of I_{Kto} . The value of half-maximal activation voltage V_h was 3.3 ± 1.3 and 3.5 ± 1.4 mV, respectively in the absence and presence of 30 $\mu\text{mol/L}$ LGST, the slope constant ϵ was 12.9 ± 1.0 and 13.3 ± 1.1 mV, respectively ($n=6$, Figure 7).

Effect of GST on steady state inactivation kinetics of I_{Kto}

Classic double pulse protocol was used to determine the voltage dependence of inactivation of I_{Kto} . Currents were elicited by a depolarizing pulse of +50 mV with 1 s preconditioning pulses from -80 to 20 mV by increment of 10 mV. The interval between two sweeps was set at 20s. A plot of normalized peak current (I/I_{max}) as a function of preconditioning potential was fitted with a Boltzmann function: $I/I_{\text{max}}=1/\{1+\exp[(V_h-V)/\epsilon]\}$, where V is the

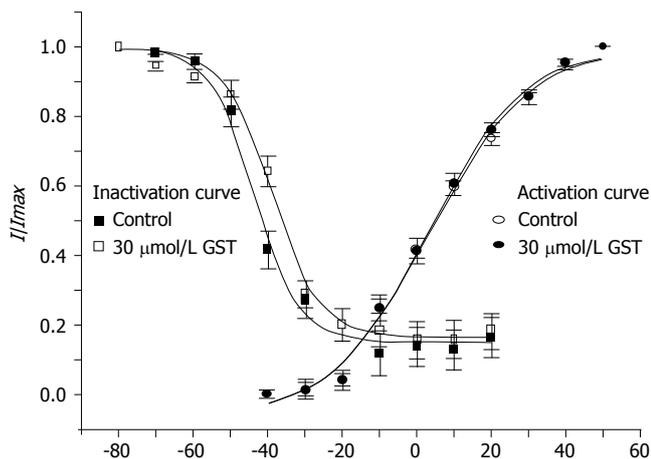


Figure 7 Effect of GST on steady state activation and inactivation of transient outward potassium currents. Inactivation is shown as a plot of normalized peak current as a function of conditional potential from -80 to +20 mV. For voltage dependence of activation, normalized currents were calculated by dividing peak outward currents by their driving force and plotted against potential. Smooth curves were fitted with Boltzmann equation which yields values of half-maximally activated or inactivated voltage (V_h) and slope constant (K).

membrane potential, V_h is the half-maximal inactivation voltage, and k is the slope constant (mV). GST had no significant effect on steady state inactivation kinetics of I_{Kto} . The values of half-maximal inactivation voltage V_h were -43.4 ± 0.7 and -39.2 ± 0.8 mV, respectively in the absence and presence of $30 \mu\text{mol/L}$ GST, the slope constant k was 5.8 ± 0.6 and 6.5 ± 0.7 mV, respectively ($n=6$, Figure 7).

VAN blocked inhibition of GST on I_{Kto}

Inhibition of phosphatase-mediated tyrosine dephosphorylation would be expected to block current inhibition by GST, since dephosphorylation in the presence of GST would require ongoing phosphatase activity^[14]. At commonly used bath concentrations of 0.1-10 mmol/L, inhibitor of tyrosine phosphatase orthovanadate could antagonize various GST-induced cellular responses such as blocking the inhibition of $I_{Ca,L}$ by PTK inhibitors^[15]. Solely applied VAN had minor stimulating but no significant effect on I_{Kto} ($n=7$). In the presence of 1 mmol/L VAN, the fractional inhibition of current f of $30 \mu\text{mol/L}$ GST significantly decreased to $28 \pm 6\%$ (Figure 1) compared with $49 \pm 6\%$ of solely applied GST ($n=10$). The rapid onset and offset responses to GST did not change (Figure 8).

DISCUSSION

At least four types of potassium channel have been identified in excitable gastrointestinal smooth muscle cells, such as voltage-dependent outward potassium channel, calcium-dependent potassium channel, ATP-sensitive potassium channel, and inward rectifier potassium channel. A given smooth muscle cell can express several families of potassium channels and several members of a single family of channels. This complexity is necessary for the control of smooth muscle function^[11]. K_v channels are of particular importance in the regulation of colonic smooth muscle electrical activity because they provide

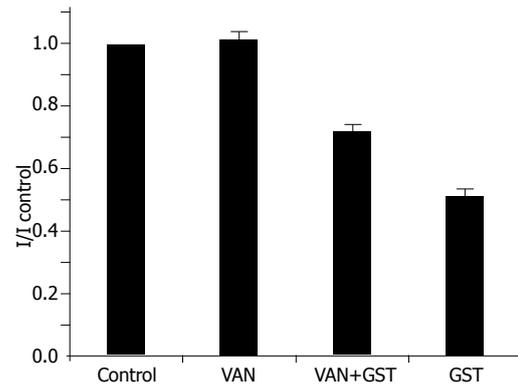


Figure 8 Orthovanadate antagonizes the inhibition of transient outward potassium currents by genistein.

outward currents over the voltage range in which these tissues operate^[11]. K_v shapes the action potential (AP) by controlling its repolarization phase and determines the membrane potential and duration of the interspike interval. Delayed rectifier potassium channels keep single AP short and permit high-frequency trains of APs. Transient outward potassium channels help a cell fire at low frequency and promote broadening of APs during repetitive activity^[16]. In murine colon, application of 4-AP to intact preparations can abolish the quiescent periods between slow waves and induce a slight depolarization^[11]. I_{Kto} is fully inactivated during the upstroke depolarization^[17]. In this study, GST could block transient outward potassium currents to depolarize membrane potential as well as delayed rectifier potassium currents to induce slight depolarization^[11], thus modulating the contraction of smooth muscle.

Protein tyrosine kinase activity is a major signaling mechanism in regulating long-term processes such as cell growth, division, and metabolism^[18]. PTK signaling is also important in regulating ion channel conductance. GST, a natural isoflavone which is abundant in soybean, is a specific inhibitor of tyrosine specific kinases by competing with ATP to form the nonproductive enzyme-substrate complexes^[19]. In this study, GST concentration - dependently and reversibly blocked voltage-gated potassium currents. Though it was reported that GST has no specific effects such as direct interaction with potassium channels^[14,20,21], orthovanadate could antagonize the blockage of GST on I_{Kto} , suggesting that although direct blockage of potassium channels by a mechanism unrelated to PTK inhibition could not be entirely excluded in this study, GST blocks the transient outward potassium channels partly via PTK pathway.

There are some differences in GST action on ion channel currents and contraction of different types of smooth muscle. GST can inhibit visceral smooth muscle contraction induced by vanadate^[8], angiotensin II^[9] and carbachol^[10] as well as nifedipine-sensitive calcium currents in rabbit colon myocytes^[22]. GST also can inhibit apamin-sensitive relaxation of the longitudinal muscle in rat distal colon induced by pituitary adenylate cyclase activating peptide^[23] as well as potassium channels^[4,5]. Besides species and tissue differences, it is possible that multiple receptor

and nonreceptor PTKs are involved in the regulation of smooth muscle contraction. Further studies are required to identify the physiological role of PTKs in gastrointestinal motility.

To check whether GST could affect the biophysical kinetics of voltage-dependent potassium channels, we examined the steady state activation and inactivation kinetics in the absence and presence of 30 $\mu\text{mol/L}$ GST. Peretz *et al.*^[4] found that GST affects potassium gating properties of Schwann cells such as a positive shift in voltage dependence of activation (by +30 mV) and a decrease in steepness of activation gating. In our experiment, no significant effect of GST on gating properties of potassium channels was observed. Whether species or cell type difference contributes to the variation needs further investigation.

In conclusion, GST concentration dependently and reversibly inhibits transient outward potassium currents.

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