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Open Channel Block of Kv3.1 Currents by Genistein, a Tyrosine Kinase Inhibitor

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The goal of this study was to analyze the effects of genistein, a widely used tyrosine kinase inhibitor, on cloned *Shaw-type* K^+ currents, Kv3.1 which were stably expressed in Chinese hamster ovary (CHO) cells, using the whole-cell configuration of patch-clamp techniques. In whole-cell recordings, genistein at external concentrations from 10 to 100 μM accelerated the rate of inactivation of Kv3.1 currents, thereby concentration-dependently reducing the current at the end of depolarizing pulse with an IC_{50} value of 15.71±0.67 μM and a Hill coefficient of 3.28±0.35 (n=5). The time constant of activation at a 300 ms depolarizing test pulses from −80 mV to +40 mV was 1.01±0.04 ms and 0.90±0.05 ms (n=9) under control conditions and in the presence of 20 μM genistein, respectively, indicating that the activation kinetics was not significantly modified by genistein. Genistein (20 μM) slowed the deactivation of the tail current elicited upon repolarization to −40 mV, thus inducing a crossover phenomenon. These results suggest that drug unbinding is required before Kv3.1 channels can close. Genistein-induced block was voltage-dependent, increasing in the voltage range (−20 mV~0 mV) for channel opening, suggesting an open channel interaction. Genistein (20 μM) produced use-dependent block of Kv3.1 at a stimulation frequency of 1 Hz. The voltage dependence of steady-state inactivation of Kv3.1 was not changed by 20 μM genistein. Our results indicate that genistein blocks directly Kv3.1 currents in concentration-, voltage-, time-dependent manners and the action of genistein on Kv3.1 is independent of tyrosine kinase inhibition.

Key Words: Genistein, Tyrosine kinase inhibitor, Kv3.1, Open channel block

INTRODUCTION

Protein kinases play an important role in modulating the activity of a variety of ion channels (Levitan, 1994; Siegelbaum, 1994; Jonas & Kaczmarek, 1996). Voltage-gated K^+ channels have been known to be involved in forming action potentials and controlling membrane excitability, neuronal firing patterns, neurotransmitter release, volume regulation and cell proliferation (Kaczmarek, 1991). While serine/threonine phosphorylation of K^+ channels by protein kinase C (PKC) and protein kinase A (PKA) is well known (Levitan, 1994; Siegelbaum, 1994; Jonas & Kaczmarek, 1996), it has recently been documented that K^+ channels are modulated by tyrosine phosphorylation via receptor and non-receptor tyrosine kinases (Holmes et al, 1996; Jonas et al, 1996; Szabo et al, 1996; Aniksztejn et al, 1997; Fadool et al, 1997). Therefore, intense research for the role of protein tyrosine kinase (PTK) in regulating ion channel activity, especially K^+ channel, is expected.

Genistein, an isoflavone compound isolated from the fermentation broth of *Pseudomonas*, has been characterized as a tyrosine kinase inhibitor because it potently and selectively exerts an inhibitory action on both receptor and non-receptor tyrosine kinases (Akiyama et al, 1987). For example, genistein was reported to inhibit the tyrosine kinase activity of $pp60^{v\text{-}src}$, however, poorly inhibit serine/threonine kinases, PKA and PKC activity (Akiyama et al, 1987; Akiyama & Ogawara, 1991). Thus, genistein has been widely used in investigating of role of PTK in the modulation pathways of various ion channel/receptor functions. Using genistein, tyrosine kinase-dependent pathway has been shown to be involved in the suppression of voltage-gated K^+ channels expressed in oocytes and HEK cells (Huang et al, 1993). In addition, genistein markedly reduces the amplitude of a slowly inactivating delayed rectifier current in cultured Schwann cells (Peretz et al, 1999). Thus, tyrosine phosphoryation could play an important role in modulating K^+ channel activities (Jonas & Kaczmarek, 1996). However, accumulating data have shown that genistein has an additional direct action on ion channels in a PTK-mediated phosphorylation-independent

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ABBREVIATIONS: CHO cells, Chinese hamster ovary cells; PTK, protein tyrosine kinase; PKC, protein kinase C; PKA, protein kinase A.

manner. Genistein inhibits directly voltage-gated K^+ channel in the pulmonary arterial cells of rats and rabbits (Smirnov & Aaronson, 1995; Washizuka et al, 1998), in the ventricular cells of guinea pig (Washizuka et al, 1998), and voltage-gated Ca^{2+} channels in the ventricular cells of guinea pig (Chiang et al, 1996). The possibility of direct blocking actions of genistein on Ca^{2+} channel in the vascular smooth muscle cells of rabbit ear artery has also been described (Wijetunge et al, 1992).

In the present study, using the patch-clamp technique, we examined the effect of genistein on the cloned rat Kv3.1 channels expressed in CHO cells, using the patch-clamp technique to determine the direct modulation of Kv3.1 channels by genistein in a phosphorylation-independent manner.

METHODS

Stable transfection and cell culture

Rat brain Kv3.1 channels stably expressed in CHO cells were used in the present study (Choi et al, 2001). The method for establishment of Kv3.1 expression in CHO cells is briefly described as follows. The cDNA of Kv3.1 (Luneau et al, 1991) was subcloned into plasmid expression vector pRc/CMV (Invitrogen Corporation, San Diego, CA, USA). CHO cells were cultured in Iscove's modified Dulbecco's medium (IMDM; Life Technologies, Grand Island, NY, USA), supplemented with 10% fetal bovine serum, 2 mM glutamine, 0.1 mM hypoxanthine and 0.01 mM thymidine, under 95% humidified air-5% CO_2 environment at 37°C. Kv3.1 expression vector was added to CHO cells for transfection using lipofectamine reagent (Life Technologies). Transfected cells were exposed to 500 μg/ml geneticin (Life Technologies), and antibiotic-resistant cells were selected and maintained in a fresh IMDM containing geneticin. By using a brief trypsin/EDTA (Life Technologies) treatment, transfected CHO cells were passed every 4~5 day and were seeded onto glass coverslips (diameter: 12 mm, Fisher Scientific, Pittsburgh, PA, USA) in a Petri dish 24 h before use. For the electrophysiological recording, cells-attached coverslips were transferred to a continually perfused (1 ml/min) recording chamber (RC-13, Warner Instrument Corporation, Hamden, CT, USA).

Electrophysiological recordings

At room temperature (22~24°C), whole-cell current of Kv3.1 was recorded and stored using the patch-clamp technique with an Axopatch 1D amplifier (Axon Instruments, Foster City, CA, USA) and a Digidata 1200A acquisition board (Axon Instruments)-equipped IBM compatible computer. Currents were sampled at 5 kHz and filtered at 2 kHz (four-pole Bessel filter). Pulse generation and data acquisition were controlled using pClamp 6.05 software (Axon Instruments). Patch electrodes were fabricated using PG10165-4 glass capillary tubing (World Precision Instruments, Sarasota, FL, USA). Liquid junction potentials between external and pipette solution were offset. Whole-cell capacitative current compensation and 80% series resistances compensation were done without any leakage compensation.

Solutions and drugs

The pipette solution contained (in mM) 140 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 EGTA and was adjusted to pH 7.3 with KOH. The bath solution contained (in mM) 140 NaCl, 5 KCl, 1.3 $CaCl_2$, 1 $MgCl_2$, 20 HEPES, and 10 glucose and was adjusted to pH 7.3 with NaOH. Genistein (Calbiochem, San Diego, CA, USA) was dissolved in dimethyl sulfoxide (DMSO, Sigma Chemical Co.) to yield 50 mM stock solutions. The concentration of DMSO in the final dilution was less than 0.1%, and this DMSO concentration had no effect on Kv3.1 currents.

Data analysis

Analysis of data was performed using pClamp 6.05 software (Axon Instruments) and Origin 6.1 software (Microcal Software, Inc., Northampton, MA, USA). The concentration-dependent curve for current block by genistein was fitted to a following equation:

$$I\ (\%) = 1/[1 + ([D]/IC_{50})^n] \quad (1)$$

in which I (%) is the percentage current block (I (%) = [1 $-I_{drug}/I_{control}] \times 100$) at test potential, [D] represents various drug concentrations, IC_{50} is a concentration of half-maximal block, and n is the Hill coefficient. Activation curves for Kv3.1 channel were fitted with a Boltzmann equation:

$$G/G_{max} = 1/[1+\exp\ (-(V-V_{1/2})/k)] \quad (2)$$

where V represents the test potential, and $V_{1/2}$ and k are the potential at which the conductance was half-maximal and slope factor, respectively. G is conductance and G_{max} is maximal conductance. Conductance was calculated by $G= I/(V-E_K)$; I is Kv3.1 current amplitude and E_K is the reversal potential of Kv3.1, which was calculated to be −85.19 mV. The steady-state voltage dependence of inactivation for Kv3.1 channel was investigated by using a two-pulse voltage protocol; currents were measured by a 300-ms test potential to +40 mV, while 20-s preconditioning pulses were varied from −60 to 0 mV stepped by 10 mV in the absence and presence of drugs. The experimental points were calculated as shown in equation 3.

$$\text{Normalized } I = (I-I_c)/(I_{max}-I_c) \quad (3)$$

in which I_{max} represents the current measured at the most hyperpolarized preconditioning pulse and I_c represents a non-zero current which was not inactivated at the most depolarized 20-s preconditioning pulse. This non-zero residual current was subtracted from the actual value. The resulting steady-state inactivation data were fitted with a Boltzmann equation:

$$y = 1/[1+\exp\ (V-V_{1/2})/k] \quad (4)$$

where V is the preconditioning potential, $V_{1/2}$ and k represents the potential corresponding to the half-inactivation point (in mV) and slope value (in mV), respectively. The steady-state voltage dependence of inactivation for Kv3.1 channel was investigated by using a two-pulse protocol; currents were measured by a 300-ms pulse to +40 mV while 20-s preconditioning pulses were varied from −60 to +10 mV stepped by 10 mV in the absence and presence

of drugs. The experimental data points were fitted as shown in equation 5.

$$I/I_{max} = 1/[1+\exp(V-V_{1/2})/k] \quad (5)$$

in which I_{max} represents the current measured at the most hyperpolarized preconditioning pulse, V is the preconditioning potential, and $V_{1/2}$ and k represent the potential corresponding to the half-inactivation point and slope factor, respectively. The time course of tail currents upon repolarization for Kv3.1 channels was fitted with a single exponential function.

Results are expressed as means±SE. Student's t-test and analysis of variance (ANOVA) were used for statistical analysis. A value of $P<0.05$ was considered statistically significant.

RESULTS

Concentration-dependent block of Kv3.1 by genistein

Fig. 1A shows the superimposed-original Kv3.1 currents expressed in CHO cells under control conditions and in the presence of various concentrations of genistein. Under control conditions, Kv3.1 currents were rapidly activated with a sigmoidal time course to a peak and then slightly inactivated during a 300-ms pulse of +40 mV, as described previously (Kanemasa et al, 1995). In the presence of genistein, blockade of Kv3.1 currents was manifested by acceleration of apparent rate of current decay without modification of activation time course. Thus, the steady-state currents at the end of the 300-ms pulse were reduced much more than the peak currents in a concentration-dependent manner. These results suggest that genistein-induced blockade of Kv3.1 currents occurred preferentially after the channels opened. Steady state of genistein- induced block of Kv3.1 was reached within 1 min and currents were restored to 93.21±2.11% of control value (n=8) within 1 min, indicating that effects of genistein were largely reversible upon washout. The concentration-dependent block of the current was measured at the end of a 300-ms pulse of +40 mV, and a nonlinear least-squares fit of the Hill equation to the individual data points yielded an IC_{50} and a Hill coefficient of 15.71±0.67 μM and 3.28±0.35 (n=5), respectively (Fig. 1B).

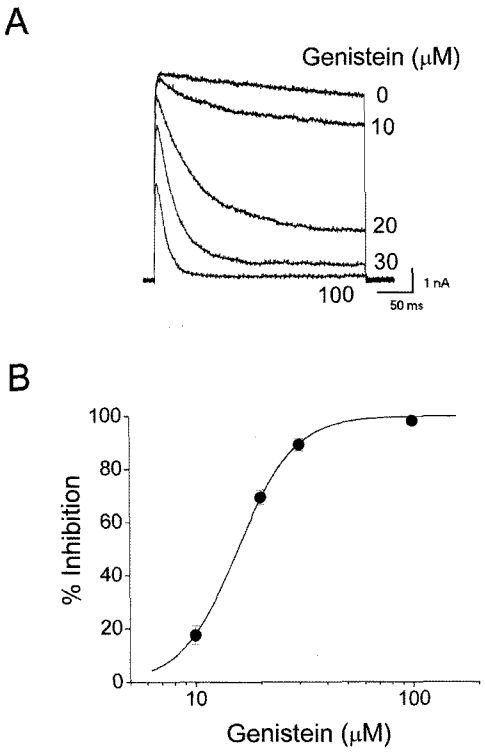

Fig. 1. Concentration dependence of genistein-induced block of Kv3.1. (A) Whole-cell Kv3.1 currents were elicited by 300 ms step depolarization to +40 mV from a holding potential of −80 mV at 10 s intervals. Control current and current following the addition of 10, 20, 30 and 100 μM genistein are indicated. (B) Concentration-response relationship for genistein block of Kv3.1. Reduction in current at the end of depolarizing steps from −80 to +40 mV was used as an index of block. Nonlinear least-squares fit of the data yielded an IC_{50} value of 15.71±0.67 μM and a Hill coefficient of 3.28±0.35 (n=5).

Effects of genistein on activation and deactivation kinetics of Kv3.1

Fig. 2A shows the superimposed original current traces of Kv3.1 obtained in the absence and presence of 20 μM genistein. Current traces were fitted by a monoexponential function to obtain an estimate of the rate of current activation. The rapid phase of current activation was not significantly modified by genistein: Namely, the time constant of activation at a 300-ms depolarizing test pulse from −80 mV to +40 mV was 1.01±0.04 ms (n=9) and 0.90±0.05 ms (n=9) under control conditions and in the presence of 20 μM genistein, respectively (Fig. 2B). Figs. 2C and 2D show the representative superimposed tail currents at the 150-ms repolarizing pulse of +40 mV after a 300-ms depolarizing pulse of +40 mV from a holding potential of −80 mV under control conditions and in the presence of 20 μM genistein. Under control conditions, the tail current declined quickly with a time constant of 3.11±0.21 ms (n=5) and nearly completely deactivated during the 150-ms repolarizing pulse of −40 mV. In the presence of 20 μM genistein, the initial peak amplitude of tail current was reduced, and the subsequent decline of the current was slower (4.37±0.27 ms, n=5, $P<0.05$), resulting in the tail crossover phenomenon.

Voltage-dependent block of Kv3.1 by genistein

Fig. 3 shows the effect of 20 μM genistein on Kv3.1 I-V relations. Under control conditions, the Kv3.1 current was activated at pulses greater than −20 mV, and I-V relationship shows a sigmoidal shape at potentials between −20 and +40 mV (Fig. 3A, 3C). The amplitude of the currents increased linearly with depolarization and reached a maximum value after a voltage step to +100 mV. These properties are same as those previously reported for Kv3.1 (Kanemasa et al, 1995; Choi et al, 2001). The block of Kv3.1 currents by 20 μM genistein was observed in the whole voltage range over which Kv3.1 was activated (Fig. 3B, 3C). By plotting % block versus potential, a high degree of block was observed with a strong voltage dependence between 0 and +30 mV, which involved the voltage range of the channel opening (Fig. 3D). Between +30 and +110 mV,

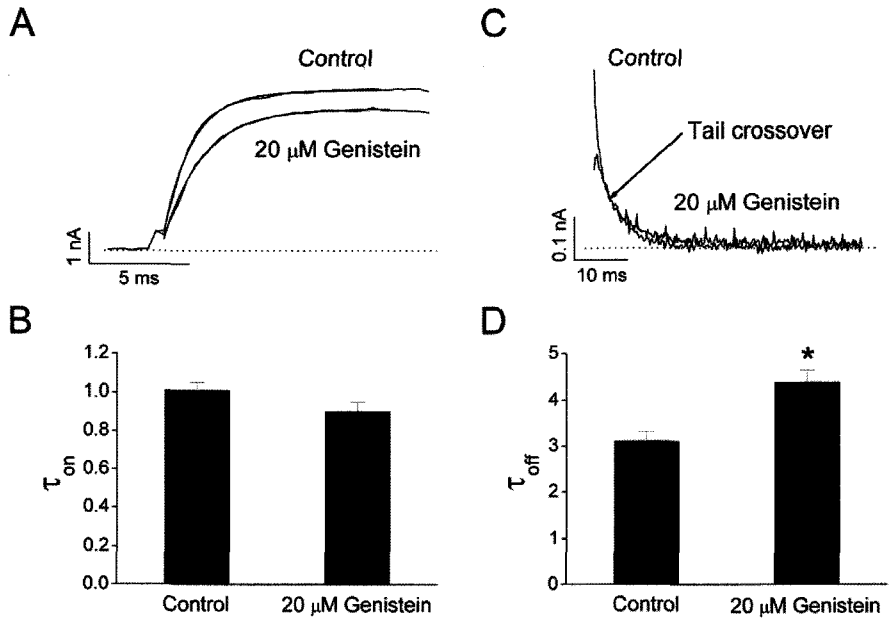

Fig. 2. Effect of genistein on activation and deactivation kinetics. (A) Superimposed original current traces under control conditions and in the presence of genistein. The solid lines over the current traces are monoexponential fits of the data. The dotted line represents zero current. (B) Summary data obtained from (A). τ_{on} represents the activation time constant. Data are expressed as means±SE (n=9). (C) Tail currents were recorded during a 250 ms repolarizing pulse of −40 mV after a 300 ms depolarizing pulse of +50 mV from a holding potential of −80 mV, in the absence and presence of genistein. By superimposing the two tail currents in the absence and presence of genistein, tail crossover phenomenon was observed. The dotted line represents zero current. (D) Summary data obtained from A. τ_{off} represents the deactivation time constants. The symbol * indicates a statistically significance ($P < 0.05$). Data are expressed as means±SE (n=5).

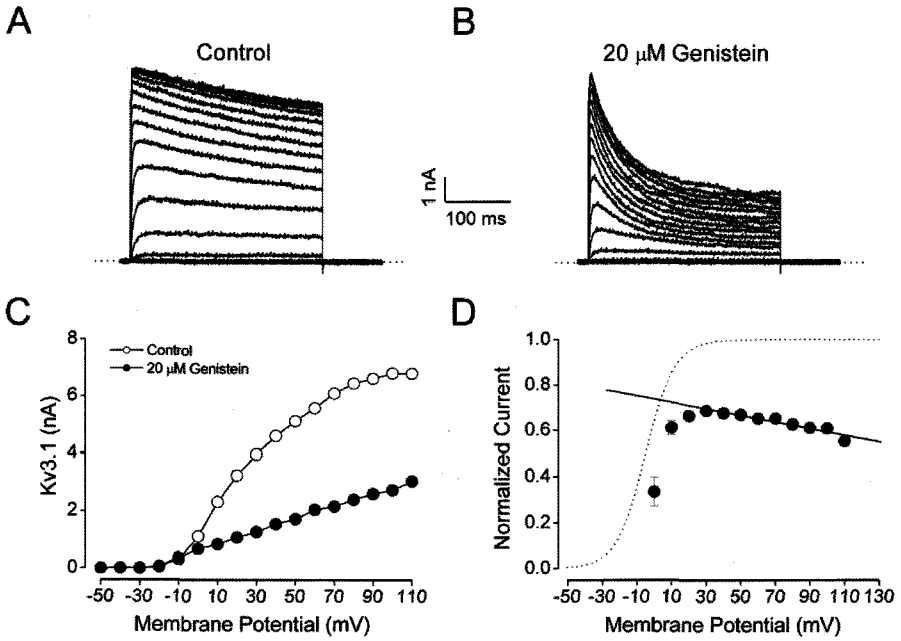

Fig. 3. Voltage-dependent block of Kv3.1 by genistein. (A) Whole- cell currents were elicited by applying 300 ms depolarizing pulses between −50 mV to +110 mV in 10 mV increments every 10 s from a holding potential of −80 mV under control conditions (A) and after the addition of 20 μM genistein (B). (C) Resulting I-V relationships taken at the end of the test pulses in the absence and presence of 20 μM genistein. (D) Normalized block from data in C. The dotted line represents the activation curve of typical Kv3.1 under control conditions. Data are expressed as means±SE (n=6).

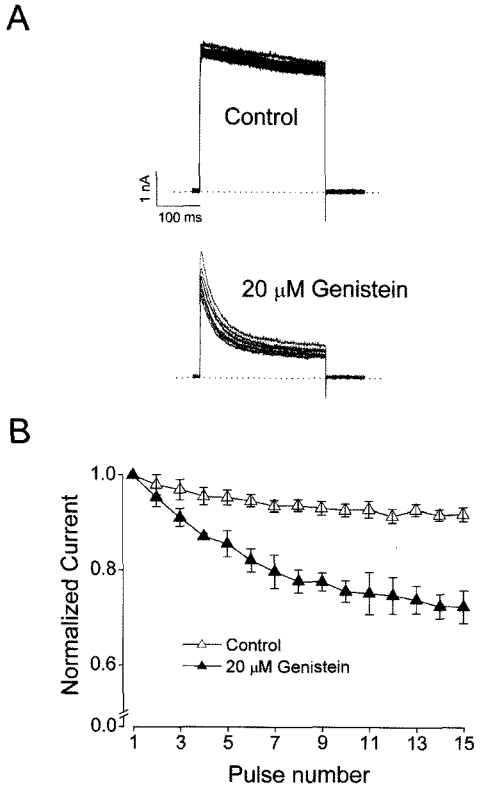

Fig. 4. Effect of genistein on use-dependent block of Kv3.1 current. (A) Original current traces obtained from applying 15 repetitive 300 ms depolarizing pulses of +40 mV from a holding potential of −80 mV at 1 Hz in the absence and presence of genistein. (B) Plot of normalized current as a function of the number of pulses. The peak amplitudes of the current at every pulse were normalized to the peak amplitude of current obtained at the first pulse. The dotted lines represent zero current. Data are expressed as means±SE (n=7).

the block decreased with a shallow voltage dependence in spite of the fact that all channels are expected to be activated over this voltage range. At +30-mV depolarizing potential, 0.3 μM BIM (I) inhibited Kv3.1 currents by 68.43 ±1.40% of control (n=6). This block decreased continuously to 55.20±2.07% (n=6) at +110 mV (ANOVA, n=5, $P<0.05$).

Use-dependent block of Kv3.1 by genistein

Fig. 4A shows the original current traces of Kv3.1 channel obtained after applying a pulse train at a frequency of 1 Hz under control conditions (upper panel) and in the presence of 20 μM genistein (down panel). Under control conditions, the peak amplitude of the Kv3.1 current decreased slightly by 13.7±3.2% (n=5) during a pulse train (Fig. 4B). In the presence of 20 μM genistein, the peak current amplitude elicited by the first depolarizing pulse was not significantly reduced, indicating an absence of tonic block. The peak amplitude of Kv3.1 thereafter progressively decreased by 56.4±7.7% (n=7) until a new steady-state block was reached (Fig. 4B). These results suggest that the block of Kv3.1 current by genistein is use-dependent.

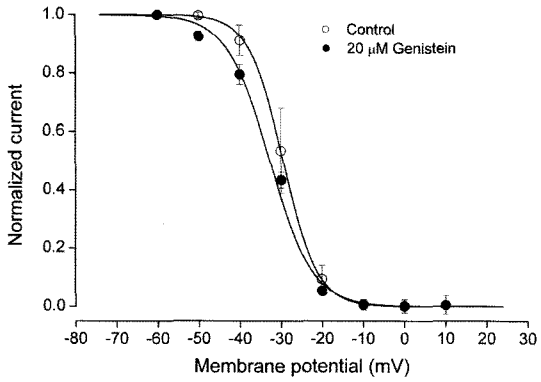

Fig. 5. Effects of genistein on the steady-state inactivation of Kv3.1. The currents were activated by a test pulse to +40 mV after a 30 s conditioning prepulse at different voltages. The peak current amplitude in the test pulse was normalized to the peak amplitude measured after a prepulse at −80 mV and plotted against the prepulse potential. Data were fitted to a Boltzmann equation. Data are expressed as means±SE (n=4).

Voltage dependence of the steady-state inactivation of Kv3.1 by genistein

Fig. 5 shows the steady-state inactivation curves of Kv3.1 under control conditions and in the presence of 20 μM genistein. The potential ($V_{1/2}$) of the half-activation point and slope factor (k) of the steady-state inactivation curves were −24.34±0.51 mV and 3.54±0.30 mV for the control, respectively, and −24.66±0.53 mV and 6.66±0.27 mV for 20 μM genistein, respectively (n=4). Thus, genistein appeared to have no effect on the voltage dependence of the steady-state activation. This lack of the effect of genistein on the voltage dependence of the steady-state inactivation suggests that genistein is unlikely to interact with the inactivated state of Kv3.1.

DISCUSSION

Kv3.1, the *Shaw-type* K^+ channel, is abundantly expressed in neurons that have the ability to fire at high frequencies (Perney et al, 1992; Weiser et al, 1994; Wang et al, 1998). Kv3.1 has a number of features distinguishable from other potassium channels: high activation threshold (−20 mV) and very rapid activation and deactivation kinetics (Grissmer et al, 1992; Kanemasa et al, 1995). Kv3.1 has about 11 putative PKC phosphorylation sites and 10 putative sites for casein kinase 2, whereas it has no site for PKA and PTK phosphorylation (Luneau et al, 1991; Macica & Kaczmarek, 2001). The absence of sites for PKA and PTK phosphorylation within Kv3.1 channel protein can usefully be exploited as a control for the study of PKA and PTK phosphorylation-involved channel modulation.

In the present study, the block of Kv3.1 by genistein was characterized by an acceleration of the apparent rate of current decay. These results are similar to those observed with various open channel blockers such as bisindolylmaleimide and norfluoxetine for rat Kv1.5 and Kv3.1 (Choi et al, 2000; Choi et al, 2001), and zatebradine, and loratadine for human Kv1.5 (Valenzuela et al, 1996; Delpon et al, 1997).

Our present results suggest that genistein blocks Kv3.1 currents by acting as an open channel blocker. First, at the onset of depolarizing pulses, genistein had no effect on the initial time course of channel activation and the peak amplitude of the current, indicating that genistein does not bind to the closed or resting state of Kv3.1. Second, genistein accelerated the rate of Kv3.1 current decay in a concentration-dependent manner during the depolarizing pulse, indicating interactions with the open state of the Kv3.1 channel. Third, the block induced by genistein was voltage-dependent since the block increased steeply in the voltage range of channel activation: The result suggests that this drug interacts preferentially with the open state of the channel. Fourth, genistein reduced the tail current amplitude and slowed the time course of the deactivating tail current, thus inducing tail crossover phenomenon: The result suggests that genistein must dissociate from its binding site before channels can close, and provides further evidence for the proposed open channel interaction. Fifth, the lack of effect of genistein on the steady-state inactivation curve suggests that genistein had no effect on the inactivated state of Kv3.1. Sixth, the action of genistein to block Kv3.1 was use-dependent, with effects enhanced at higher rates of channel activation. This is consistent with the action of genistein on the open state of Kv3.1 (Snyders & Yeola, 1995; Valenzuela et al, 1996; Delpon et al, 1997; Choi et al, 2001).

Genistein, a potent and specific inhibitor of protein tyrosine kinase, is one of the major bioactive isoflavones abundant in some vegetables, and has been reported to exert a wide range of biological actions. For instance, genistein reduces proliferation and induces apoptotic death in colon cancer cell (Yu et al, 2004), and has antiproliferative effect on several types of cancer cell lines (Su et al, 2003; Chang et al, 2004; Su et al, 2005). Although these results suggest that genistein may be of use as an anticancer agent, its mechanisms of action and its molecular targets on cancer cell remain unclear. Voltage-gated K^+ channels are expressed widely in a variety of cells and have been reported to be involved in the proliferation of many types of cells including tumor cell lines (Dubois & Rouzaire-Dubois, 1993; Wang, 2004). These above cited studies suggest that K^+ channels could be considered as a potential therapeutic target for cancer therapy. Although clinical significance is not clear, our result that genestein blocked Kv3.1 provides potentially promising information about the application of genistein to cancer chemotherapy.

In conclusion, we suggest that the block of Kv3.1 currents by genistein does not occur via a phosphorylation-dependent signal transduction pathway but rather as an open channel blocker via a direct one-to-one interaction between the drug and the channels.

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