

# Genistein inhibits voltage-gated sodium currents in SCG neurons through protein tyrosine kinase-dependent and kinase-independent mechanisms

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**Abstract** Voltage-gated sodium channels play a crucial role in the initiation and propagation of neuronal action potentials. Genistein, an isoflavone phytoestrogen, has long been used as a broad-spectrum inhibitor of protein tyrosine kinases (PTK). In addition, genistein-induced modulation of ion channels has been described previously in the literature. In this study, we investigated the effect of genistein on voltage-gated sodium channels in rat superior cervical ganglia (SCG) neurons. The results show that genistein inhibits  $\text{Na}^+$  currents in a concentration-dependent manner, with a concentration of half-maximal effect ( $\text{IC}_{50}$ ) at  $9.1 \pm 0.9 \mu\text{M}$ . Genistein positively shifted the voltage dependence of activation but did not affect inactivation of the  $\text{Na}^+$  current. The inactive genistein analog daidzein also inhibited  $\text{Na}^+$  currents, but was less effective than genistein. The  $\text{IC}_{50}$  for daidzein-induced inhibition was  $20.7 \pm 0.1 \mu\text{M}$ . Vanadate, an inhibitor of protein tyrosine phosphatases, partially but significantly reversed genistein-induced inhibition of  $\text{Na}^+$  currents. Other protein tyrosine kinase antagonists such as tyrphostin 23, an erbstatin analog, and PP2 all had small but significant inhibitory effects on  $\text{Na}^+$  currents. Among all active and inactive tyrosine kinase inhibitors tested, genistein was the most potent inhibitor of  $\text{Na}^+$  currents. These results suggest that genistein inhibits  $\text{Na}^+$  currents in rat SCG neurons through two distinct

mechanisms: protein tyrosine kinase-independent, and protein tyrosine kinase-dependent mechanisms. Furthermore, the Src kinase family may be involved in the basal phosphorylation of the  $\text{Na}^+$  channel.

**Keywords** Genistein · Voltage-gated sodium channels · SCG · Inhibition · Tyrosine · Phosphorylation

## Introduction

Voltage-gated sodium channels (VGSC) play a critical role in the generation and propagation of action potentials in excitable cells including neurons, muscle cells, and endocrine cells. VGSC are composed of a complex of a  $\alpha$  subunit that forms the voltage-sensitive and ion-selective pore, in association with one or more auxiliary  $\beta$  subunits. To date, nine  $\alpha$  subunits of the VGSC superfamily,  $\text{Na}_v1.1$ – $\text{Na}_v1.9$ , have been identified, which are widely distributed in mammalian tissue (see review [10] and [37]). Mutations in VGSC have been associated with a number of neurological diseases including inherited epilepsy and pain conditions and have been implicated in various to psychiatric disorders (see review [21]).

Within the VGSC family,  $\text{Na}_v1.1$ ,  $\text{Na}_v1.2$ ,  $\text{Na}_v1.3$ , and  $\text{Na}_v1.6$  are distributed in central nervous system (CNS) neurons and  $\text{Na}_v1.7$ ,  $\text{Na}_v1.8$ , and  $\text{Na}_v1.9$  are selectively distributed in peripheral nervous system (PNS) neurons.  $\text{Na}_v1.1$ ,  $\text{Na}_v1.2$ ,  $\text{Na}_v1.3$ ,  $\text{Na}_v1.6$ , and  $\text{Na}_v1.7$  are highly tetrodotoxin (TTX) sensitive whereas  $\text{Na}_v1.8$  and  $\text{Na}_v1.9$  are TTX resistant (see review [10] and [14]). Thus, TTX-sensitive VGSC currents in SCG neurons are most likely  $\text{Na}_v1.7$  currents.

Genistein, an isoflavone phytoestrogen, known as a potent and specific inhibitor of protein tyrosine kinases

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(PTK) [1], has been reported to modulate ion channel function through PTK-dependent and PTK-independent mechanisms. A PTK-independent effect was considered for genistein-induced inhibition of L-type  $\text{Ca}^{2+}$  currents in rat and guinea pig ventricular cells [36, 5],  $\text{K}^{+}$  currents in guinea pig ventricular cells [32],  $^{22}\text{Na}^{+}$  influx and  $\text{Na}^{+}$  currents in rat cerebellar granule cells [24], fast sodium currents in uterine leiomyosarcoma cells [19], and pacemaker ( $I_f$ ) currents in rabbit sinoatrial node cells [3]. On the other hand, a PTK-dependent mechanism was suggested for genistein-induced inhibition of L-type  $\text{Ca}^{2+}$  channel activity in pituitary GH3 cells [9], ATP-sensitive  $\text{K}^{+}$  channels in rabbit portal vein smooth muscle cells [23], fast sodium currents in rabbit ventricular myocytes [31], slowly delayed-rectifier  $\text{K}^{+}$  currents ( $I_k$ ) in mouse Schwann cells [25], and guinea pig ventricular myocytes [22].

To date, there have been no studies investigating the role of genistein on the function of sodium channels from PNS neurons such as the SCG. Furthermore, no information is available regarding the role of tyrosine phosphorylation in the functional modulation of  $\text{Na}^{+}$  channels such as  $\text{Na}_v1.7$ . In this paper, we investigate the effect of genistein on  $\text{Na}^{+}$  currents in rat SCG neurons and demonstrate that genistein inhibits  $\text{Na}^{+}$  current via two distinct mechanisms: a protein tyrosine kinase-independent and protein tyrosine kinase-dependent pathway.

## Materials and methods

### Chemicals

The drugs and chemicals used in this experiment were obtained as follows: Genistein, daidzein, erbstatin analog, tyrphostin 23, PP2, Perorthovanadate, 5, 4-[2-hydroxyethyl]-1-piperazineethane-sulfonic acid (HEPES), Dulbecco's modified Eagle's medium (DMEM), dimethyl sulfoxide (DMSO), were purchased from Sigma-Aldrich (St Louis, MO, USA). TTX was purchased from Swellxin Science and Technology (Guangzhou, China). Perorthovanadate ( $\text{Na}_3\text{VO}_4$ ) in the bath solution was prepared by a 20-min incubation with the oxidant  $\text{H}_2\text{O}_2$  (0.003%) and subsequent quenching of residual  $\text{H}_2\text{O}_2$  with 100 U/ml catalase for 5 min [16].

### Cell culture

Primary cultures of neurons were prepared from SCG from 3- to 5-week-old Sprague–Dawley rats using a previously described procedure [17]. Briefly, rats were killed by cervical dislocation and ganglia were rapidly removed from the carotid bifurcation and placed in modified D-Hanks' solution. Ganglia were digested at 37°C with collagenase (1 mg/ml, Worthington) and dispase (5 mg/ml, Sigma-

Aldrich) for 30 min, followed by another 30-min digestion with trypsin (2.5 mg/ml, Worthington). They were subsequently resuspended at least three times in DMEM medium plus 10% bovine calf serum (Hy-Clone, USA) to stop the digestion. Ganglia were then dissociated into a suspension of individual cells and planted on poly-D-lysine coated glass coverslips in 24-well tissue culture plates (Costar, USA). Cells were incubated at 37°C with a 5%  $\text{CO}_2$  and 95% air atmosphere. The medium was changed to neurobasal A medium plus 2% B27 supplement (Invitrogen) after 12 h and neurons were used for recording within 48 h. Within this time frame, no processes developed in these neurons. Procedures for the primary culture of neurons from the hippocampus and cortex were similar to SCG neurons, except these neurons were digested at 37°C with trypsin (1.25 mg/ml, Worthington) for 12 min.

### Electrophysiology

Perforated-patch whole-cell recordings were used in this study. Recordings were made at room temperature (20–25°C). Pipettes were pulled from borosilicate glass capillaries and had a resistance of 3–5  $\text{M}\Omega$  when filled with internal solution. Series resistance compensation has always been used. Normally up to 80–90% compensation can be reached in our condition. Under this condition, the maximum access resistance is about 2  $\text{M}\Omega$ . Currents were recorded using Axon patch 200B amplifier and pClamp 9.0 software (Axon Instruments, CA), and were filtered at 10 KHz. The voltage protocol used to study Na currents of SCG neurons was as follows: the cells were held at  $-70$  mV and 20 ms depolarizing step to 0 mV was applied every 3 s. The current-clamp technique was used to record the action potentials of SCG neurons. The action potentials were elicited by an approximate twofold threshold depolarizing current of 0.1 nA. The sampling rate was 10 KHz for membrane currents and was 2.5 KHz for membrane potential recordings. Compounds were delivered to the cell through a home-made perfusion pipette which was placed just beside the cell. The bath chamber was continuously superfused with background control solution. For the perforated patch recording, a pipette was first front-filled with the standard internal solution, then backfilled with the same internal solution containing amphotericin B (120 ng/ml). The external solution used to record the Na currents from SCG neurons contained (in mM): NaCl 120, KCl 3, HEPES 5,  $\text{NaHCO}_3$  23, glucose 11,  $\text{MgCl}_2$  1.2,  $\text{CaCl}_2$  2.5,  $\text{BaCl}_2$  0.2, and  $\text{CdCl}_2$  0.2 (adjusted to pH 7.4 with NaOH). The internal solution for sodium current recording consisted of (in mM): CsCl 90, KCl 40, HEPES 20, and  $\text{MgCl}_2$  3 (adjusted to pH 7.3–7.4 with CsOH). The external solution used to record neuronal action potentials contained (in mM): NaCl 120, KCl 3, HEPES 5,  $\text{NaHCO}_3$  23, glucose 11,  $\text{MgCl}_2$  1.2, and  $\text{CaCl}_2$  2.5, (adjusted to

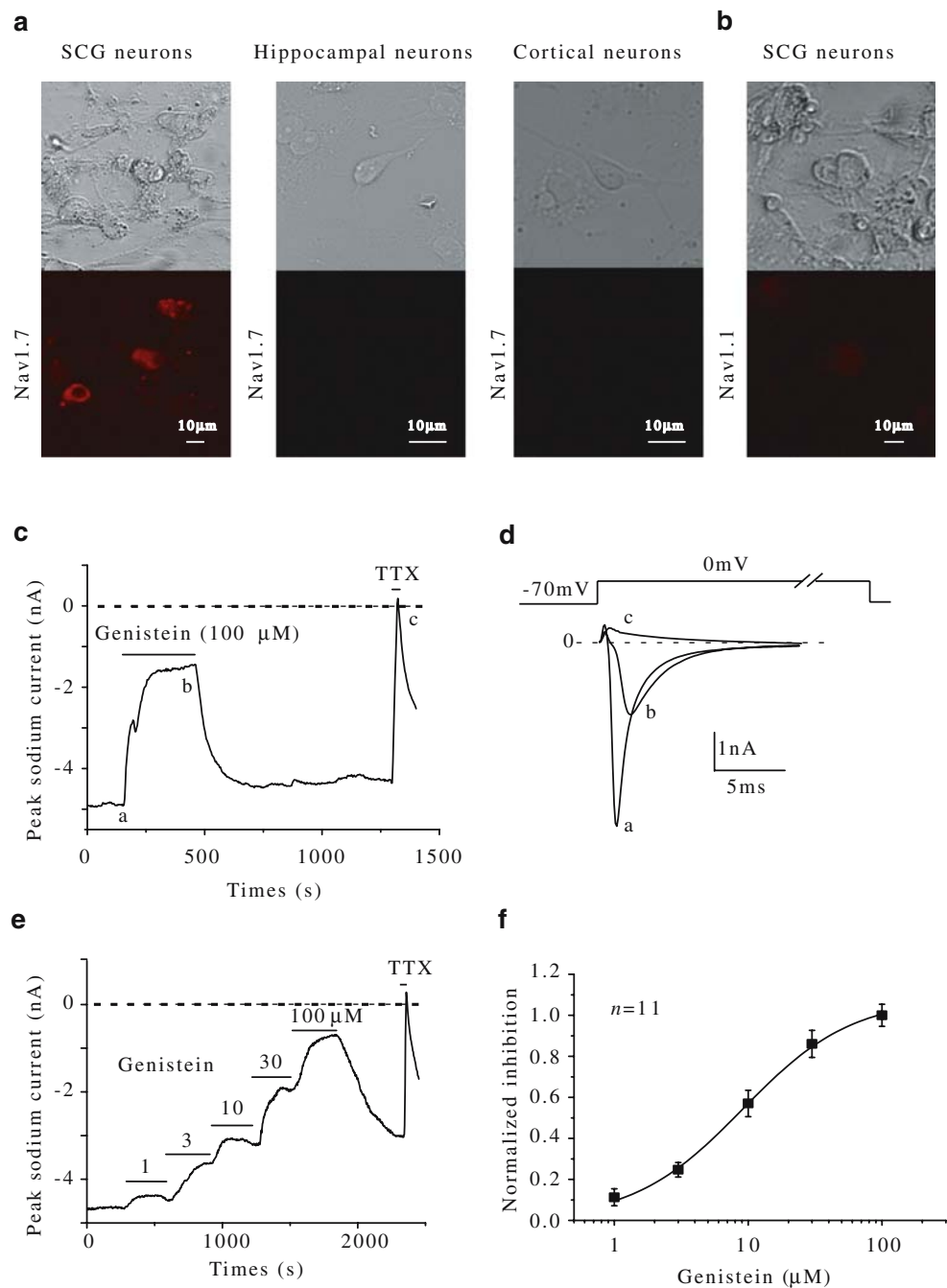
pH 7.4 with NaOH). The internal solution for action potential recording contained (in mM): KAC 90, KCl 40, HEPES 20, and MgCl<sub>2</sub> 3 (adjusted to pH 7.3–7.4 with KOH).

### Cell immunofluorescence and confocal imaging

Neurons were seeded on poly-lysine coated coverslips and were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked with 3% BSA for 30 min before incubation for labeling. The cells were washed three

times with 0.01 M phosphate buffered saline (PBS, pH 7.4) before each of the above treatments. Subsequently, neurons were incubated with primary rabbit anti-Na<sub>v</sub>1.7 Ab or anti-Na<sub>v</sub>1.1 Ab (1:200, Santa Cruz Biotechnology) in blocking solution overnight at 4°C, followed by three washes with PBS and incubation with goat anti-rabbit IgG-TRITC secondary Ab (1:100, Chemicon) for 30 min at 37°C. Cells were examined on an inverted laser-scanning microscope (SP2, Leica, Germany). TRITC was excited at 564 nm and the emitted fluorescence signal was at 570 nm.

**Fig. 1** Genistein inhibits Na<sup>+</sup> currents in rat SCG neurons. **a** Immunofluorescence of Na<sub>v</sub>1.7 channel in rat SCG neurons. The lower panel shows confocal images of SCG, hippocampal, and cortical neurons probed with an anti-Na<sub>v</sub>1.7 antibody and TRITC-conjugated anti-rabbit antibody, respectively. The upper panel shows phase-contrast microscopy of the same cells (scale bars, 10 μm). **b** Immunofluorescence of Na<sub>v</sub>1.1 channel in rat SCG neurons (scale bars, 10 μm). **c** Perforated-patch recording of Na<sup>+</sup> currents in rat SCG neurons. Cells were held at -70 mV and Na<sup>+</sup> current were elicited by depolarization to 0 mV for 20 ms every 3 s. Bath application of genistein (100 μM) for 5 min induced a bi-phasic inhibition of Na<sup>+</sup> current. TTX (0.05 μM) abolished the Na<sup>+</sup> current. **d** Representative traces of Na<sup>+</sup> current elicited at 0 mV and taken from the corresponding time shown in **c**. **e** Concentration-dependent inhibition of Na<sup>+</sup> current by genistein. **f** Concentration–response relationship for genistein. The data was fitted by the Hill function which produced an IC<sub>50</sub> of 9.1±0.9 μM and a coefficient of 1.1 (n=11). Error bars represent SEM



## Statistics

The programs “Origin” (Version 7.0, OriginLab Corporation) and Excel (Microsoft) were used for data analysis. Data were presented as the mean±SEM. Statistical significance was computed using Student’s *t* test. The differences were considered significant if  $p < 0.05$ .

## Results

### Genistein inhibits Na<sup>+</sup> currents in rat SCG neurons in a concentration-dependent manner

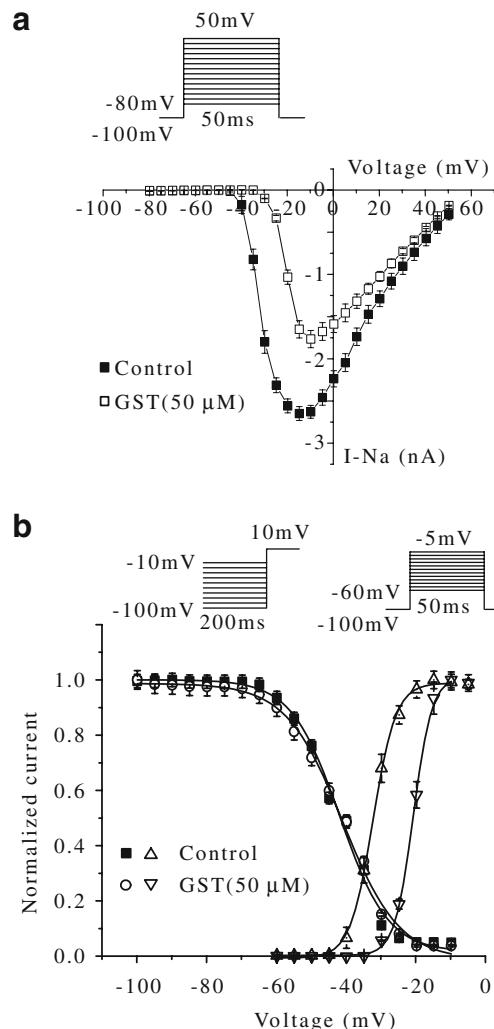
As discussed in the Introduction, the TTX-sensitive Na<sup>+</sup> current in SCG neurons most likely comes from Na<sub>v</sub>1.7. Indeed, immunocytochemistry revealed the presence of Na<sub>v</sub>1.7 channels in SCG neurons (Fig. 1a). We also tested for the presence of the Na<sub>v</sub>1.1 channel in SCG neurons (Fig. 1b), which was previously reported to be present at lower levels in DRG neurons [4]. Similarly, we also detected a very weak signal for Na<sub>v</sub>1.1 in SCG neurons (Fig. 1b). To confirm the specificity of the channel-antibody, hippocampal and cortical neurons were used in control experiments and no immunoreactivity for Na<sub>v</sub>1.7 was found in these neurons (Fig. 1a). Thus, TTX-sensitive Na<sub>v</sub>1.7 currents were the dominant Na<sup>+</sup> currents in SCG neurons. The maximal Na<sup>+</sup> current amplitude is about 5 nA, but most are around 3.5 nA.

We then tested the effect of genistein on Na<sup>+</sup> currents in SCG neurons. Genistein (100 μM), when applied to the bath, induced an inhibition of peak Na<sup>+</sup> current by 73.3%±5.4% ( $p < 0.01$ ,  $n = 12$ ) (Fig. 1c,d). The Na<sup>+</sup> current almost fully recovered when genistein was washed out. Maximal inhibition was obtained within 195.9±17.7 s of genistein application ( $n = 12$ ) (Fig. 1c), which was much longer than the reported inhibition of sodium currents in CNS neurons by genistein [24]. It is interesting to note that two phases of inhibition were evident for most cases (Fig. 1c): an initial fast inhibition followed by a relatively slow inhibition. These two processes were fitted with a double exponential function and time constants were obtained. The fast time constant was 10.6±1.2 s ( $n = 6$ ) and the slow time constant was 55.9±2.4 s ( $n = 6$ ), respectively. Genistein (0.1~100 μM) inhibited peak Na<sup>+</sup> currents in a concentration-dependent manner (Fig. 1e,f). Genistein could inhibit Na<sup>+</sup> currents at concentrations as low as 0.3 μM and reached maximal inhibition at a concentration of 100 μM (Fig. 1e,f). We did not use genistein at concentrations higher than 100 μM due to its poor solubility at these concentrations. The concentration–response curve for genistein was well-described by the Hill equation with a concentration for half-maximum inhibition (IC<sub>50</sub>) of 9.1±0.9 μM and a coefficient

of 1.1±0.2 ( $n = 11$ ) (Fig. 1f). TTX (0.05 μM) could rapidly and completely inhibit the Na<sup>+</sup> current (Fig. 1c,e).

### Genistein inhibits voltage-dependent activation but not inactivation of the Na<sup>+</sup> current in SCG neurons

The effects of genistein (50 μM) on the current–voltage relationship and the voltage-dependence of activation and inactivation of the Na<sup>+</sup> current were tested (Fig. 2). Neurons were held at -100 mV and Na<sup>+</sup> currents were



**Fig. 2** Effect of genistein on current–voltage curve, activation, and inactivation of Na<sup>+</sup> current. **a** Current–voltage (*I*–*V*) curve of Na<sup>+</sup> currents recorded from SCG neurons. The neurons were held at -100 mV and depolarized for 50 ms from -80 to 50 mV in 5-mV steps. Genistein (50 μM) was applied for 5 min. Filled square and open square indicated before and after genistein application, respectively. **b** Effects of genistein (50 μM) on voltage-dependent activation and inactivation of Na<sup>+</sup> currents. Filled square/open triangle, before and open circle/open inverted triangle, after application of genistein for 5 min, respectively. Steady-state inactivation curves were elicited by a 200-ms prepulse from -100 to -10 mV in 5-mV steps, and the activation curves were elicited by a test pulse from -60 to -5 mV in 5-mV steps. Error bars represent SEM

evoked by depolarization for 50 ms from  $-80$  mV to  $+50$  mV in 5-mV steps to obtain the current–voltage relationship curve ( $I$ – $V$  curve) (Fig. 2a). Genistein clearly reduced peak  $\text{Na}^+$  currents. The effect of genistein on the voltage-dependent activation and inactivation of  $\text{Na}^+$  currents is shown in Fig. 2b. The protocols used to elicit voltage-dependent activation and inactivation of the  $\text{Na}^+$  currents are shown in the inset of Fig. 2b. Genistein significantly shifted the voltage-dependent activation of  $\text{Na}^+$  current to the right, but did not affect voltage-dependent inactivation (Fig. 2b). The half-maximal activation potential ( $V_{1/2}$ ) was significantly more positive for genistein-treated neurons ( $-20.6 \pm 0.1$  mV,  $n=9$ ) than in control cells ( $-32.4 \pm 0.2$  mV,  $n=9$ ). The half-maximal inactivation potentials were  $-41.3 \pm 0.4$  mV ( $n=9$ ) for genistein and  $-41.8 \pm 0.3$  mV ( $n=9$ ) for controls, respectively.

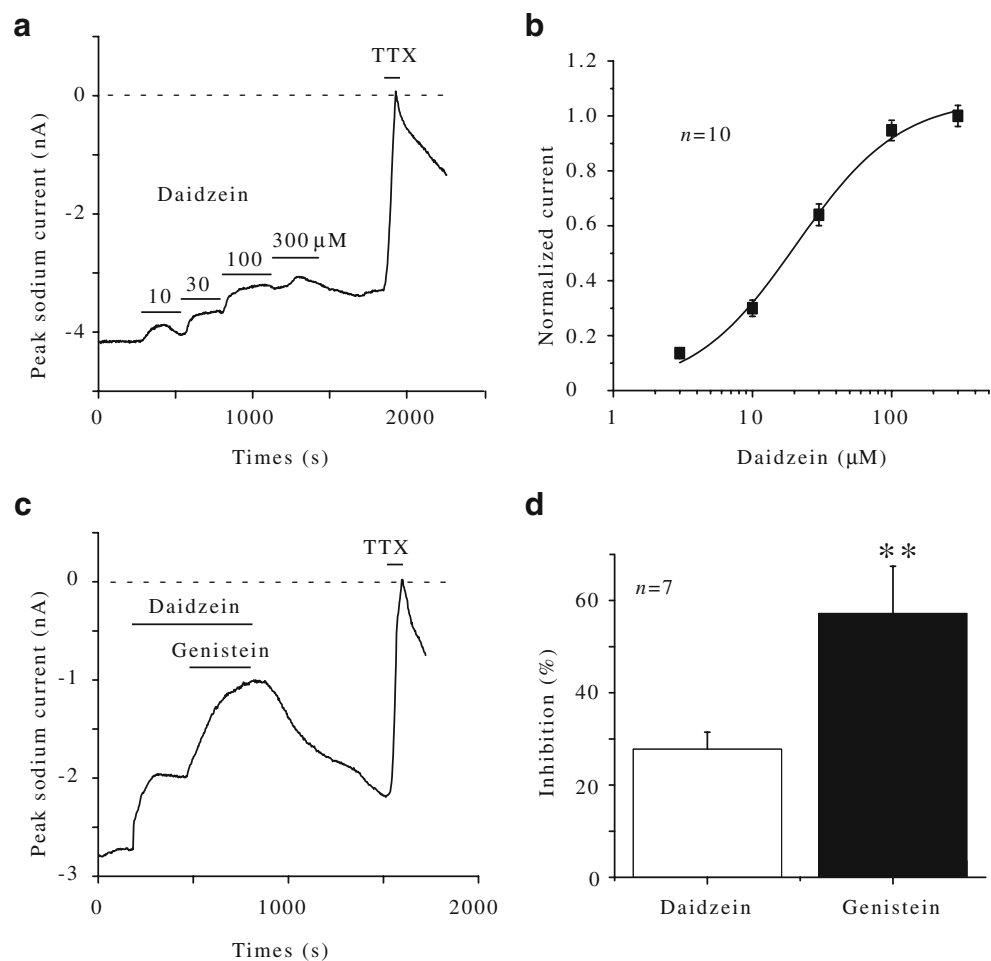
Genistein-induced inhibition of  $\text{Na}^+$  currents involves two mechanisms: PTK-independent and PTK-dependent mechanisms

It was previously shown that genistein inhibits sodium currents in CNS neurons through its direct interaction with

channels and not through tyrosine kinase inhibition [24]. To investigate the underlying mechanisms involved in genistein-induced inhibition of  $\text{Na}^+$  currents in rat SCG neurons, we tested daidzein, an inactive structural analog of genistein with no activity against protein tyrosine kinases. Daidzein rapidly inhibited  $\text{Na}^+$  currents in a concentration-dependent manner (Fig. 3a). Maximal inhibition was reached at a concentration of  $100 \mu\text{M}$ , which reduced the  $\text{Na}^+$  peak current by  $28.5\% \pm 3.1\%$  ( $n=10$ ) (Fig. 3a,b). The Hill fitting of the concentration–response data produced an  $\text{IC}_{50}$  of  $20.7 \pm 0.1 \mu\text{M}$  and a coefficient of  $1.2 \pm 0.2$  ( $n=10$ ) (Fig. 3b).

To compare the effects of genistein and daidzein, experiments were conducted in the same cell with the maximal inhibitory concentrations of  $100 \mu\text{M}$  for both agents (Fig. 3c,d). Daidzein ( $100 \mu\text{M}$ ) was first applied for 5 min and then genistein ( $100 \mu\text{M}$ ) was applied for another 5 min (Fig. 3c) in the continuous presence of daidzein. Daidzein alone and genistein in the presence of daidzein reduced peak  $\text{Na}^+$  currents by  $27.8\% \pm 3.6\%$  and  $57.3\% \pm 10.1\%$  ( $p < 0.01$ ,  $n=7$ ), respectively (Fig. 3c,d). Thus, at the same maximal concentration, the inhibition produced by daidzein was substantially smaller. Furthermore, daidzein-

**Fig. 3** Daidzein inhibits  $\text{Na}^+$  currents in rat SCG neurons. **a** Daidzein was applied at various concentrations (10, 30, 100, 300  $\mu\text{M}$ ) for the indicated period of time. **b** Concentration–response relationship of daidzein-induced inhibition on  $\text{Na}^+$  current. The data was fitted by the Hill function.  $\text{IC}_{50}$  is  $20.7 \pm 0.1 \mu\text{M}$  and coefficient is 1.2 ( $n=10$ ). **c** Representative time course of  $\text{Na}^+$  current in the presence of daidzein ( $100 \mu\text{M}$ ) alone and in the presence of both daidzein and genistein ( $100 \mu\text{M}$ ). **d** Summary data for genistein- and daidzein-induced inhibition of  $\text{Na}^+$  currents (\*\* $p < 0.01$ ,  $n=7$ ). Error bars represent SEM



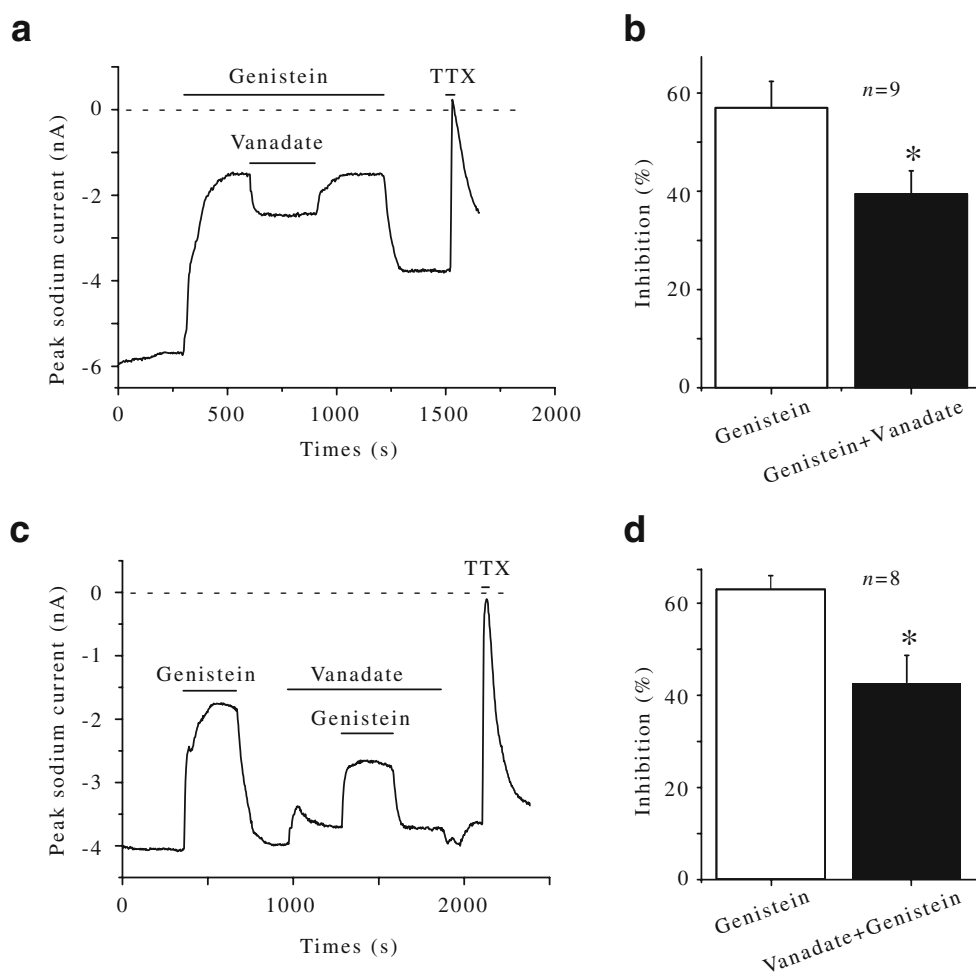


only-induced inhibition of  $\text{Na}^+$  currents was faster than genistein-induced inhibition (Fig. 3c). Since daidzein is an inactive structural analog of genistein, these data suggest that part of genistein-induced inhibition is through a PTK-independent mechanism. The data also suggest that genistein utilizes additional mechanisms since genistein is much more potent than daidzein in inhibiting  $\text{Na}^+$  currents (Fig. 3d).

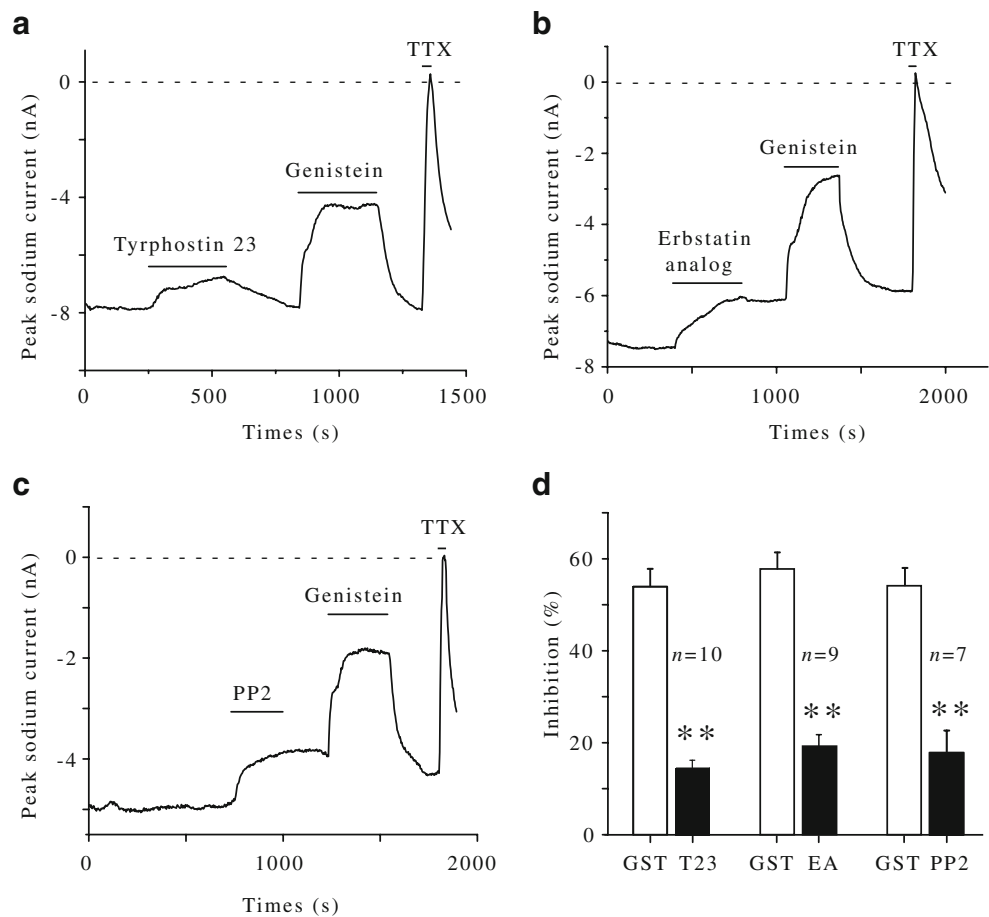
Several lines of evidence have shown that genistein modulates ion channels through a PTK-dependent mechanism (e.g., [9, 23, 25, 31, 22]). To determine whether inhibition of PTK activity is involved in genistein-induced inhibition of  $\text{Na}^+$  currents, we first used sodium orthovanadate ( $\text{Na}_3\text{VO}_4$ ), a potent inhibitor of protein tyrosine phosphatases (PTP). Vanadate (1 mM) partially rescued  $\text{Na}^+$  currents from genistein-induced inhibition (Fig. 4a), reversing genistein-induced inhibition of  $\text{Na}^+$  currents from  $57\% \pm 5.4\%$  to  $39.5\% \pm 4.7\%$  ( $p < 0.05$ ,  $n = 9$ ) (Fig. 4b). Pretreatment with vanadate reduced genistein-induced inhibition of  $\text{Na}^+$  currents by  $18.7\% \pm 3.3\%$  ( $n = 8$ ) (Fig. 4c,d). Thus, inhibition of PTK activity is also partly responsible for the observed  $\text{Na}^+$  current inhibition by genistein.

We then tested the effects of other PTK inhibitors such as tyrphostin 23, an erbstatin analog, and PP2. Among these three potent inhibitors of tyrosine kinases, the erbstatin analog and PP2 are also regarded as selective inhibitors for the Src kinase family, and tyrphostin 23 is, similar to genistein, a broad-spectrum PTK inhibitor [22]. To get a good comparison between these PTK inhibitors and genistein, genistein was used with each of these three PTK inhibitors in the same cell (Fig. 5a–c). Tyrphostin 23, the erbstatin analog, and PP2 all inhibited  $\text{Na}^+$  current in SCG neurons. The inhibition was smaller compared to the effect of genistein, but significant. Tyrphostin 23 (100  $\mu\text{M}$ ), Erbstatin analog (100  $\mu\text{M}$ ), and PP2 (1  $\mu\text{M}$ ) inhibited  $\text{Na}^+$  currents by  $14.6\% \pm 0.5\%$  ( $p < 0.05$ ,  $n = 10$ ),  $19.3\% \pm 1.0\%$  ( $p < 0.01$ ,  $n = 9$ ), and  $17.8\% \pm 1.9\%$  ( $p < 0.01$ ,  $n = 7$ ), respectively, whereas genistein (100  $\mu\text{M}$ ) inhibited  $\text{Na}^+$  currents by 54–58% (Fig. 5a–d). It should be noted that tyrphostin 23-induced inhibition of  $\text{Na}^+$  current was almost completely reversible, whereas the inhibition produced by the erbstatin analog and PP2 was irreversible. We also noticed that a fast component of inhibition was always present with genistein-induced inhibition, whereas it

**Fig. 4** Vanadate partly reduces genistein-induced inhibition of  $\text{Na}^+$  current. **a** Vanadate (1 mM) partly reverses the genistein (100  $\mu\text{M}$ )-induced inhibition of  $\text{Na}^+$  current. **b** Summary data for **a** ( $*p < 0.05$ ,  $n = 9$ ). **c** Pretreatment with vanadate (1 mM) reduced genistein (100  $\mu\text{M}$ )-induced inhibition of  $\text{Na}^+$  current. **d** Summary data for **c** ( $*p < 0.05$ ,  $n = 8$ ). Error bars represent SEM



**Fig. 5** PTK inhibitors inhibit  $\text{Na}^+$  currents. Tyrphostin 23 (T23, 100  $\mu\text{M}$ ) (a), erbstatin analog (EA, 100  $\mu\text{M}$ ) (b), and PP2 (1  $\mu\text{M}$ ) (c) all inhibited  $\text{Na}^+$  current to a similar degree, which was significantly less than genistein (GST, 100  $\mu\text{M}$ )-induced inhibition of  $\text{Na}^+$  current (d) (\*\* $p < 0.01$ ,  $n = 10$  for Tyrphostin 23,  $n = 9$  for Erbstatin analog and  $n = 7$  for PP2). Error bars represent SEM



was absent during inhibition with the other three PTK inhibitors (Fig. 5a–c). Furthermore, the characteristic of the slow component in genistein-induced inhibition was similar to that of inhibition by other PTK inhibitors. It is worth noting that the concentrations of the tyrosine kinase inhibitors used here are much higher than those normally used (10  $\mu\text{M}$  for tyrphostin 23 [23], 10  $\mu\text{M}$  for erbstatin analog [24], and 0.2  $\mu\text{M}$  for PP2 [13]). We used high concentrations of these PTK inhibitors to get a full assessment of the PTK mechanisms involved in genistein-induced inhibition of  $\text{Na}^+$  current. The combined data suggest that about one-third of the genistein-induced inhibition of  $\text{Na}^+$  currents occurs through inhibition of tyrosine phosphorylation.

#### Genistein suppresses excitability in rat SCG neurons

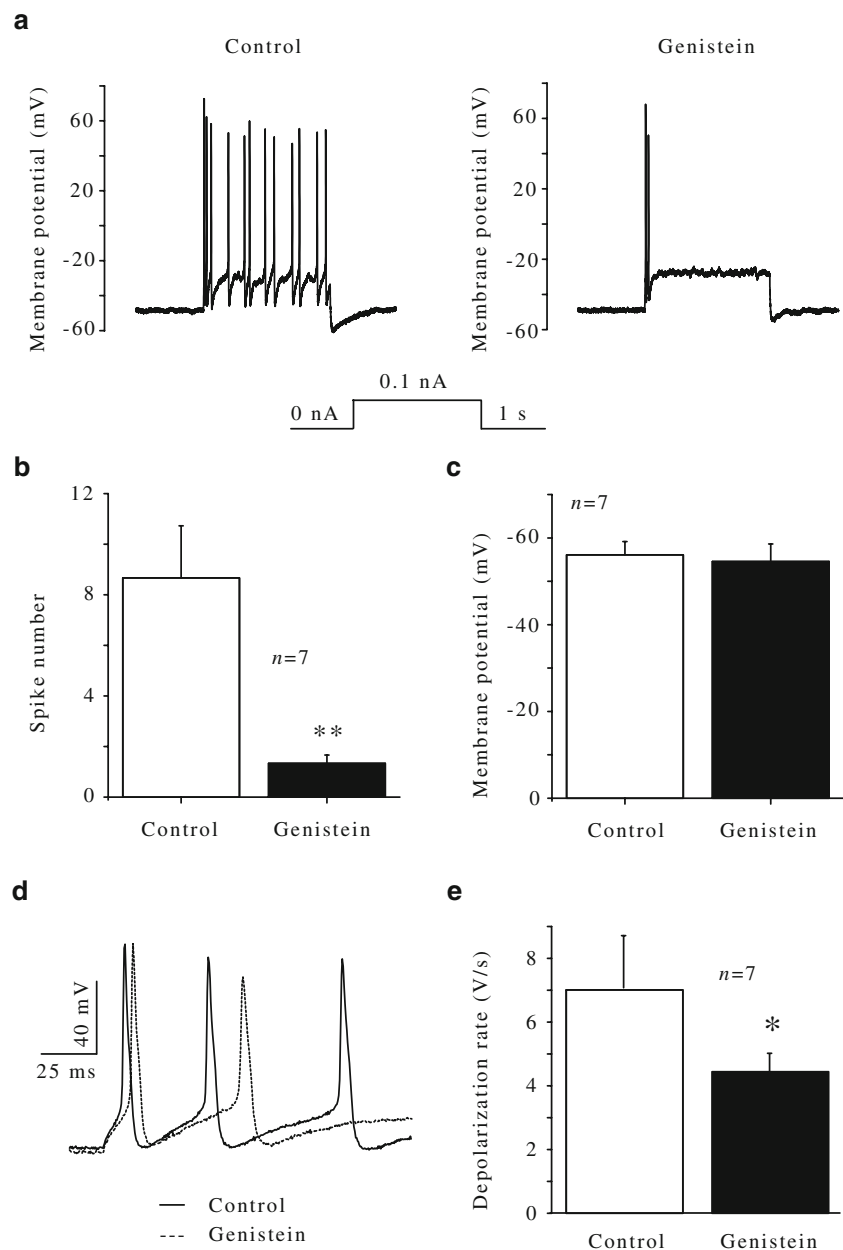
It is well known that VGSCs play a critical role in the generation and propagation of action potentials. Modulation of sodium channel function will inevitably alter the excitability of excitable cells. To confirm that inhibition of  $\text{Na}^+$  current by genistein reduces neuronal excitability, we performed current-clamp patches to record action potentials elicited by current injection (see the detail in material and

methods). Bath application of genistein (100  $\mu\text{M}$ ) immediately suppressed the excitability of SCG neurons (Fig. 6a), by reducing the number of action potentials from  $8.7 \pm 2.1$  to  $1.3 \pm 0.3$  ( $p < 0.01$ ,  $n = 7$ ) (Fig. 6b). However, genistein did not alter the resting membrane potential ( $56.1 \pm 3.1$  and  $54.7 \pm 4.1$  mV before and after application of genistein, respectively) (Fig. 6c). We also quantified the depolarization rate of action potentials before and after the application of genistein (Fig. 6d,e). Genistein significantly reduced the depolarization rate of action potentials from  $7.0 \pm 1.3$  to  $4.4 \pm 0.9$  V/s ( $p < 0.01$ ,  $n = 7$ ) (Fig. 6e).

#### Discussion

Genistein is an isoflavone phytoestrogenic molecule with three hydroxyl substituents [34]. Interest in genistein has been raised due to its reported anticancer and cardioprotective properties [2, 38]. Genistein is a frequently used agent in cell signaling studies including studies of ion channel modulation, although some of genistein's effects on ion channels have been attributed to PTK-independent mechanisms. In the present study, we demonstrated that

**Fig. 6** Genistein suppresses the excitability in rat SCG neurons. **a** Current clamp was used to record the action potentials of rat SCG neurons. Cells were held at 0 pA and injected with 100 pA current for 2 s to elicit action potentials. The *left* and *right panels* show the results before and after the application of genistein (100  $\mu$ M), respectively. **b** Summary data show the effects of genistein (100  $\mu$ M) on the spike number of action potentials (\*\* $p$ <0.01,  $n$ =7). **c** Summary data show the effect of genistein (100  $\mu$ M) on the resting membrane potential ( $n$ =7). **d** Superimposed action potentials in the control (*solid line*) and in the presence of genistein (*dashed line*) (100  $\mu$ M). **e** Summary data for the depolarization rate before and after genistein (100  $\mu$ M) application. Error bars represent SEM



genistein inhibits voltage-sensitive sodium currents in rat SCG neurons through both PTK-independent and PTK-dependent mechanisms.

Several lines of evidence support that genistein inhibits  $\text{Na}^+$  currents through two distinct mechanisms. First, the time course of genistein-induced inhibition has two phases, an initial fast phase and a subsequent slow phase, which can be described by a double exponential function with time constants of  $10.6 \pm 1.2$  and  $55.9 \pm 2.4$  s, respectively. These time course characteristics are consistent with a PTK-independent fast effect and a slow process involving alteration of phosphorylation. Second, daidzein, an inactive structural analog of genistein, as well as three other PTK

inhibitors tyrphostin 23, an erbstatin analog, and PP2, all inhibited  $\text{Na}^+$  currents but to a less degree than genistein. In a quantitative estimation, two-thirds of genistein-induced inhibition may come from a PTK-independent effect (based on ~30% inhibition by daidzein) and one-third may come from the inhibition of PTK activity (based on ~20% inhibition by tyrphostin 23, erbstatin analog and PP2). Finally, vanadate, presumably through an increase of phosphorylation by the inhibition of phosphatases, reversed or prevented genistein-induced inhibition. Again, ~20% reduction in inhibition by genistein in the presence of vanadate is consistent with our above quantitative estimation. However, it is possible that the two distinct mechanisms have a



synergistic effect since the summed effect of daidzein and other modulators of tyrosine phosphorylation (~50%) are somewhat smaller than the effect of genistein (~55–70%).

Sodium channels in cultured CNS neurons are the targets for cAMP-dependent protein kinase (PKA) and protein kinase C (PKC) [20]. Many physiological signals such as dopamine and acetylcholine have been shown to regulate sodium currents in CNS neurons through phosphorylation [7, 8, 29, 33]. Sodium currents also have been shown to be modulated by tyrosine phosphorylation or dephosphorylation. For example, tyrosine phosphorylation induced by Src kinase reduces sodium currents and causes a negative shift in channel inactivation in PC12 cells in the presence of nerve growth factor or other growth factors [15]. Tyrosine dephosphorylation of sodium channels produced by receptor protein tyrosine phosphatase  $\beta$ , which is associated with sodium channels, increases the whole-cell sodium current, slows sodium channel inactivation, and positively shifts its voltage dependence in tsA-201 cells and rat brain cells [26]. However, it is not clear if basal tyrosine phosphorylation of sodium channels plays an important role in the modulation of sodium channel function in neurons. In the present study, our results indicate that basal phosphorylation in resting conditions plays an important role in the function of voltage-gated sodium channels in rat SCG neurons, since all modulators of tyrosine phosphorylation affect channel function.

Previous work has shown that genistein directly inhibits  $^{22}\text{Na}^+$  influx through neurotoxin-activated sodium channels in brain neurons and  $\text{Na}^+$  currents in cerebellar granule cells [24] and inhibits fast sodium currents in uterine leiomyosarcoma cells [19] in a PTK-independent manner. However, it was also reported that genistein inhibits fast sodium currents in rabbit ventricular myocytes through a PTK-dependent mechanism [31]. Our data in the present study suggest that  $\text{Na}^+$  currents in rat SCG neurons are inhibited by genistein through PTK-dependent as well as PTK-independent mechanisms. Two explanations can be provided for this difference. The first difference may arise from the different  $\text{Na}^+$  channel isoforms involved in the studies. PNS neurons including DRG [18, 27] and SCG [6, 30] neurons predominantly express  $\text{Na}_v1.7$  channels whereas cerebellar granule cells predominantly express  $\text{Na}_v1.2$  and  $\text{Na}_v1.6$  channels [28]. Sodium currents recorded in muscle cells are most likely from  $\text{Na}_v1.4$  and  $\text{Na}_v1.5$  channels [14, 37]. The second possibility is differences in cellular environments where  $\text{Na}^+$  channels are modulated. In other words, the basal phosphorylation process in different cells could be quite different.

In the present study, we utilized rat SCG neurons as a platform for recording  $\text{Na}^+$  currents mainly because they highly express  $\text{Na}_v1.7$  channels [6, 30] (Fig. 1a). The  $\text{Na}_v1.7$  channel has been suggested to play an important

role in pain pathways. For example, a missense mutation of the  $\text{Na}_v1.7$  coding gene, *SCN9A*, leads to primary erythralgia [12, 35], an inheritable human pain condition characterized by intermittent pain, redness, heat, and swelling in the extremities [11]. To our knowledge, our data is the first to suggest that  $\text{Na}_v1.7$  channels are targeted for modulation through phosphorylation. It would be interesting to know whether  $\text{Na}_v1.7$  channel phosphorylation plays a role in physiological and pathophysiological conditions.

Sodium current is a major determinant for neuronal excitability [14]. Modulation of neuronal sodium channels will inevitably alter excitability. Thus, it is not surprising that genistein rapidly suppressed neuronal excitability and the depolarization rate of action potentials in the present study. However, the resting membrane potential was not altered, indicating that potassium currents which contribute mostly to the resting membrane potential are not affected by genistein.

Genistein has been widely used as a blocker of tyrosine kinases and has been used to study signaling mechanisms. The present study and many other reports have suggested its involvement in PTK-independent mechanisms on ion channel functions. However, we emphasize that caution is warranted when a mechanism underlying ion channel modulation is established based on the effect of genistein. Both a PTK-independent effect and an effect through tyrosine phosphorylation must be considered carefully.

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