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Rapid report

Genistein inhibition of fast Na^+ current in uterine leiomyosarcoma cells is independent of tyrosine kinase inhibition

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Abstract

Possible regulation of fast Na^+ channels by tyrosine kinase was examined in a human uterine smooth muscle cell line, using whole-cell voltage clamp (at a holding potential of −90 mV). Bath application of genistein, an inhibitor of tyrosine kinase, decreased the fast Na^+ current ($I_{Na(f)}$) dose-dependently. The maximal inhibition of $I_{Na(f)}$ was 98%, and the concentration for half-maximal inhibition (IC_{50}) was 9 μM. The effect of genistein was rapidly reversible. Daidzein, an inactive analog of genistein, had a similar inhibitory effect on $I_{Na(f)}$. These results suggest that the fast Na^+ channels in uterine sarcoma cells may be directly blocked by genistein and daidzein, i.e., their effect may be independent of tyrosine kinase inhibition.

Keywords: Tyrosine kinase; Genistein; Daidzein; Leiomyosarcoma cell; Sodium ion current; Whole-cell voltage clamp; (Uterine smooth muscle)

Voltage-dependent fast Na^+ channels may play a key role in excitability and propagation of uterine smooth muscle during labor [1]. Protein phosphorylation (on serine and/or threonine groups) is a major regulatory process for the modulation of ion channels such an L-type Ca^{2+} channels in neuronal cells, myocardial cells, and vascular smooth muscle cells [2,3]. In uterine smooth muscle cells, cAMP and cGMP (and their respective protein kinases) do not modulate the L-type Ca^{2+} current ($I_{Ca(L)}$) [1], whereas protein kinase C (PKC) increases $I_{Ca(L)}$ [4]. There are also some reports about the modulation of the fast Na^+ channels by cAMP [5] or PKC [6]. However, in myometrial cells, cAMP, cGMP, and PKC do not appear to modulate the fast Na^+ current ($I_{Na(f)}$) (Kusaka, M. and Sperelakis, N., unpublished data). To examine the possible modulation of Na^+ channels by tyrosine-specific protein kinase in uterine cells, we examined the effect of genistein, an inhibitor of tyrosine protein kinase (TPK).

Tyrosine kinase has been shown to be correlated with the signal transduction for several growth factors, such as epidermal growth factor (EGF) [7], fibroblast growth factor (FGF) [8], platelet-derived growth factor (PDGF) [9], and for insulin [10]. In neuronal cells, these growth factors were reported to cause expression of fast Na^+ channels in neurons [11], basic FGF to increase $I_{Ca(L)}$ [12,13]. TPK inhibitors, such as genistein, were reported to block the Ca^{2+} current in vascular smooth muscle cells [14], and to activate a non-selective cation channel [15]. However, no reports have been published about the regulation of the fast Na^+ current ($I_{Na(f)}$) by TPK.

In this study, we investigated the possible modulation of the fast Na^+ channels by TPK in human uterine leiomyosarcoma cells by studying the effect of TPK inhibitors using the whole-cell patch clamp. The leiomyosarcoma cell line (SK-UT-1B), derived from human uterine myometrial cells, was used for the present study because it is a good model for the uterine smooth muscle cells of late-pregnant rats. It was demonstrated that the rat myometrial cells gain fast Na^+ channels during late pregnancy, which may play an important role in normal labor [16,17]. We discovered that the leiomyosarcoma cell also exhibits $I_{Na(f)}$, under the control of one or more factors in serum [18]. In addition, $I_{Na(f)}$ was almost 10-fold greater in density in the leiomyosarcoma cell, compared to the pregnant rat myometrial cell. We found that $I_{Na(f)}$ is inhibited by both the TPK inhibitor, genistein, and its inactive analog, daidzein. Thus, endogenous TPK activity may not be involved in the regulation of Na^+ channel activity in myometrial cells.

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The human leiomyosarcoma cell line (SK-UT-1B) was obtained from American Type Culture Collection (Rockville, MD). The cells were kept frozen in small aliquots at −80°C. To start cell growth, some aliquots were transferred to culture flasks (25 cm^2 bottom), and the cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS), 100 U/ml penicillin G, and 100 μg/ml streptomycin (Gibco Laboratories, Grand Island, NY). The cell cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air, and grown until confluency. The cells were passaged every two weeks. For passage, confluent monolayers were dissociated from the flasks using phosphate-buffered saline containing the proteolytic enzyme, dispase (0.5 mg/ml) (Boehringer Mannheim, Indianapolis, IN). The culture medium was changed every 2–3 days for cell 'feeding'. For patch-clamp experiments, cells were plated on coverslips and incubated in medium for 24–72 h.

Whole-cell voltage clamp recording was carried out with a suction pipette and a patch clamp amplifier (Axopatch-1D, Axon Instruments, Foster City, USA) using standard techniques. The patch electrodes (2–5 MΩ) were made from borosilicate glass capillary tubing (World Precision Instruments, USA). The cell suspension was placed into a small chamber (0.2 ml) on the stage of an inverted microscope (TMD-Diaphoto, Nikon, Tokyo, Japan). To isolate $I_{Na(f)}$, the pipette was filled with high Cs^+ solution of the following composition (in mM): 110 CsOH, 20 CsCl, 110 glutamic acid, 5 $MgCl_2$, 10 Hepes, 5 Na_2ATP, 10 EGTA (pH 7.2). The bath solution contained (in mM): 140 NaCl, 2 $NiCl_2$, 10 glucose, 10 Hepes, 3 4-aminopyridine (pH 7.35).

Series resistance was partly (ca. 80%) compensated electrically. Leak current and residual capacitive current were subtracted using the P/4 protocol. Current and voltage signals were filtered at 2 kHz and digitized by an A/D converter (TL-1, Axon Instruments), and analyzed on an IBM-AT personal computer using the pCLAMP software (Axon Instruments). The membrane capacitance was determined from the current amplitude elicited in response to a hyperpolarizing voltage ramp pulse of 0.2 V/s from a holding potential (HP) of 0 mV to avoid interference by any time-dependent ionic currents. All experiments were carried out at room temperature (22–24°C).

Genistein and daidzein were obtained from Calbiochem-Novabiochem Corp. (San Diego, CA, USA). These agents were dissolved in dimethylsulfoxide (DMSO) for a stock solution, and aliquots frozen until use. The final maximal concentration of DMSO (0.1%) had no effect on $I_{Na(f)}$.

Data are presented as means ± S.E. Theoretical curves were fitted to the data with the least-squares method.

The mean membrane capacitance of the sarcoma cells was 37.1 ± 2.4 pF ($n = 34$), and it was not affected by application of genistein or daidzein. Unless stated otherwise, the $I_{Na(f)}$ was elicited by 20 ms depolarizing step pulses to 0 mV from a HP of −90 mV every 20 s. The study of the effect of genistein was started when $I_{Na(f)}$ became stabilized after breaking into the cell. The current was completely inhibited by tetrodotoxin (TTX) (data not shown), and the kinetic properties including inactivation time-course and current–voltage relationship were consistent with those of $I_{Na(f)}$ we reported previously for uterine smooth muscle cells [16,17].

Table 1
Effects of genistein and daidzein on $I_{Na(f)}$ in uterine leiomyosarcoma cells (SK-UT-1B)

	Peak $I_{Na(f)}$ (% of control)
Genistein 100 μM	8.3 ± 1.4 (6)
Daidzein 100 μM	19.0 ± 3.6 (5)

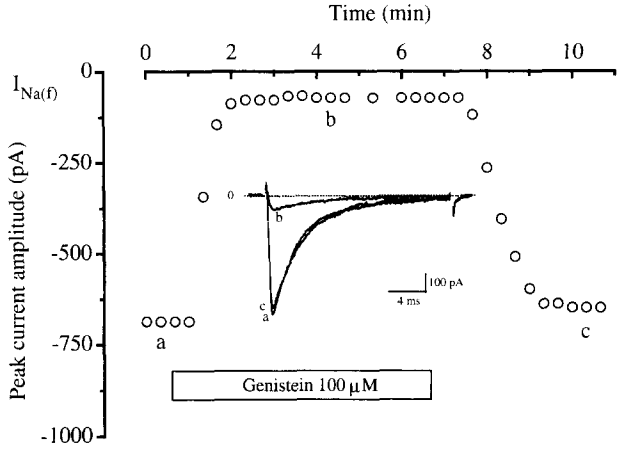

Fig. 1. Inhibition of $I_{Na(f)}$ by genistein (100 μM) in a human uterine leiomyosarcoma cell. The effect reached a stable level within 2 min after bath application of genistein, and was rapidly reversed by washout. Currents were elicited from a HP of −90 mV to a step potential of 0 mV every 20 s. (Inset) Superimposed current traces showing the $I_{Na(f)}$ recorded before (a) and 4 min after (b) application of genistein, and after washout (c).

Bath application of genistein (100 μM) reduced $I_{Na(f)}$ by 91.7 ± 1.4% ($n = 6$) (Table 1). Fig. 1 shows the time-course of the effect of genistein on peak current amplitude in one representative experiment. The decrease in $I_{Na(f)}$ started within 1 min after application of genistein, and reached a stable level within 2 min. The effect of genistein on $I_{Na(f)}$ was almost completely reversed by washout. Superimposed current traces before (a) and 4 min after (b) genistein application, and after washout (c) are given in the inset. The current–voltage relationship for peak $I_{Na(f)}$ was not significantly shifted by genistein (data not shown); there was no obvious change in voltages for threshold or peak current.

Fig. 2 shows the dose–response curve for the inhibitory effect of genistein. Peak $I_{Na(f)}$ amplitude in the presence of genistein was plotted against the various concentrations of

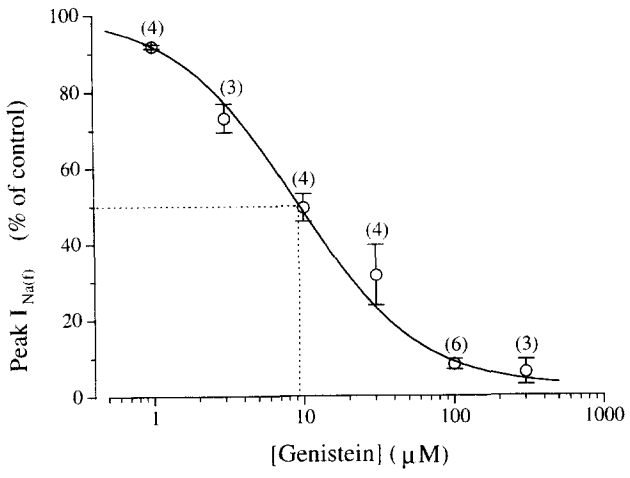

Fig. 2. The dose–response relationship for genistein inhibition of $I_{Na(f)}$ in human uterine leiomyosarcoma cells. Peak $I_{Na(f)}$ amplitude was measured before and 4 min after application of various concentrations of genistein in the bath. Pulse protocol was the same as in Fig. 1. Numbers in parentheses indicate the number of cells for each data points. Points plotted are the mean ± S.E. The IC_{50} value was 9.0 μM.

the drug as a percent of control. Data points were fitted to the Hill equation: $I_{genistein}/I_{control}$ (%) $= E_{max}/[1 + ([genistein]/IC_{50})^{nH}] + (100 - E_{max})$, where E_{max} is the maximum inhibitory effect, n_H is the Hill coefficient, IC_{50} is the concentration of the drug causing 50% of the maximal inhibitory effect. The n_H value was 1.1, and the IC_{50} value was 9.0 μM. The maximum inhibitory effect was calculated to be 98.0%.

In order to examine whether the effect of genistein on $I_{Na(f)}$ was mediated by TPK, experiments were performed using daidzein, which has a similar structure to genistein, but has no inhibitory effect on TPK [19]. Bath application of 100 μM daidzein decreased $I_{Na(f)}$ almost as much as genistein (Fig. 3). The mean percent decrease of the peak

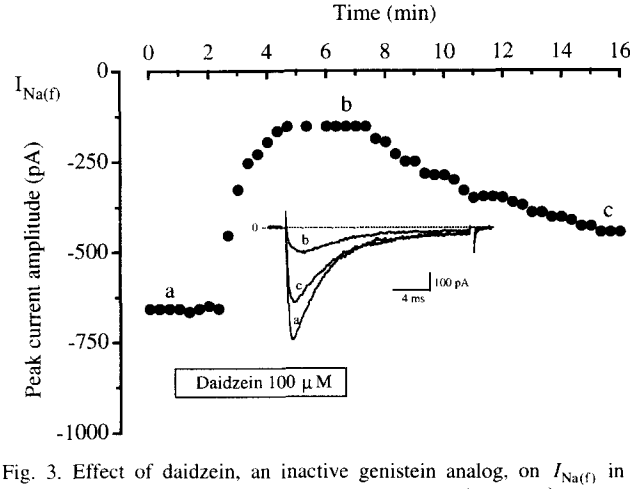

Fig. 3. Effect of daidzein, an inactive genistein analog, on $I_{Na(f)}$ in a leiomyosarcoma cell. Bath application of daidzein (100 μM) produced marked inhibition of the current. The experimental protocol was the same as in Fig. 1. (Inset) Superimposed current traces recorded during the control period (a), in the presence of daidzein (b), and after washout (c).

$I_{Na(f)}$ produced by daidzein was 81.0 ± 3.6% ($n = 5$) (Table 1). Two other concentrations of daidzein were tested and the percent decrease in the peak current was 6.0 ± 1.8% ($n = 3$) at 1 μM and 40.6 ± 6.0% ($n = 3$) at 10 μM.

The time course of recovery of $I_{Na(f)}$ upon washout of the drugs was sometimes very fast (e.g., within 2 min) and complete (e.g., Fig. 1), whereas in some experiments it was slower and incomplete (e.g., Fig. 3). In this respect, there was no difference between the two drugs; i.e., the recovery was variable with both genistein and daidzein.

In the present study, we found that a selective TPK inhibitor, genistein, inhibits $I_{Na(f)}$ dose-dependently in human uterine leiomyosarcoma cells. The maximal inhibition was nearly 100%, and the IC_{50} value was 9 μM. The onset of the inhibition of $I_{Na(f)}$ produced by genistein was fast (within a few minutes), and the effect could be quickly washed out. The inactive genistein analog, daidzein, also produced a similar decrease of the current. These results suggest that $I_{Na(f)}$ inhibition produced by genistein may not be mediated by inhibition of endogenous TPK activity, but rather may be a direct effect on the ion channel. We believe this to be true, although the concentration range of genistein that affected $I_{Na(f)}$ in this study ($IC_{50} = 9$ μM) is comparable to that needed for the inhibition of tyrosine kinases [19]. The Hill coefficient of 1.1 (see Fig. 2) suggests that one drug molecule is sufficient to block one ion channel.

A direct blocking action of genistein on the Na^+ channels is likely. Genistein and daidzein had similar potent inhibitory effects on $I_{Na(f)}$. The IC_{50} values of these agents for the inhibition of EGF receptor kinase were reported to be 2.6 μM and > 370 μM, respectively [19]. Therefore, at the concentrations we used in this study (1–100 μM), daidzein should not affect TPK. Consistent with our findings, both active and inactive tyrphostin derivatives were reported to inhibit $I_{Ca(L)}$ in vascular smooth muscle cells [14]. This provides evidence for a direct action of these agents on the ion channel proteins. In addition, the rapid onset of the effect of genistein and the rapid offset (washout recovery) may suggest a direct action on the outer surface of the channel. Since the maximum degree of inhibition of $I_{Na(f)}$ was almost 100% (Fig. 2), this is consistent with a site of action directly on the channel protein.

In contrast to the present results, we found that, in late-pregnant rat myometrial cells, genistein inhibited $I_{Ca(L)}$ only to about 50% of control, and daidzein had no effect [20]. We concluded, therefore, that the genistein inhibition of the $I_{Ca(L)}$ was mediated by inhibition of the TPK activity, and that there was a tonic stimulation of the Ca^{2+} channels by phosphorylation with TPK. In contrast, the present experiments suggest that the fast Na^+ channels are not likely to be modulated by phosphorylation with TPK, and that the drug acts as a direct blocker of the Na^+ channels.

In summary, genistein inhibits $I_{Na(f)}$ in human uterine leiomyosarcoma cells. This effect may not be mediated by

inhibition of TPK, but may be a direct action on the Na^+ channels. The present results showing that genistein inhibits $I_{Na(f)}$ at a concentration similar to that required for inhibition of TPK activity, should serve as a warning that such drugs may exert more than one action. Therefore, investigators making use of such enzyme inhibitors must be made aware and exercise caution accordingly.

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