Direct Block of Voltage-Sensitive Sodium Channels by Genistein, A Tyrosine Kinase Inhibitor

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ABSTRACT

Genistein, an isoflavone inhibitor of tyrosine-specific protein kinases, was shown to specifically block the $^{22}\text{Na}^+$ influx through voltage-sensitive Na$^+$ channels in cultured rat brain neurons, whereas other tyrosine kinase antagonists such as lavendustin A, compound 5, tyrphostin A$_47$, and an erbstatin analog were inactive at concentrations known to block kinase activity in other neuronal systems. Dose-response curves for genistein indicated a half-maximum effect at 60 nM. Analysis of Na$^+$ currents by the whole-cell recording technique showed that 20 µM genistein reduced the sodium current and shifted the voltage dependence of both activation and inactivation curves. No competition with [3H]batrachotoxinin A 20-α-benzoate to rat brain synaptosomal membranes was partially inhibited, which suggested a direct or allosteric interaction with neurotoxin binding site 2. These data taken together clearly indicate that the inhibition of voltage-sensitive sodium channels by genistein is not mediated by tyrosine kinase inhibition.

In this paper, we demonstrate that genistein also blocks voltage-sensitive Na$^+$ channels in cultured neurons. Because sodium channel activity in cultured central nervous system neurons is modulated by cAMP-dependent protein kinase and protein kinase C (Li et al., 1993), one could not exclude that genistein-induced channel blockade could be a consequence of the inhibition of the channel phosphorylation by a tyrosine kinase. However, our results clearly suggest that the blockade is caused by direct interaction of genistein with the channel protein.

Materials and Methods

Materials. [3H]STX (63 Ci/mmol) was from Amersham (Arlington Heights, IL); $^{22}\text{Na}Cl$ and [3H]BTX-B (50 Ci/mmol) were from Dupont New England Nuclear (Boston, MA). Toxin II from the scorpion Androctonus australis Hector (α-ScTx) was a generous gift from Prof. H. Rochat (Marseille, France). The pyrethroid RU39568 was from Roussel-Uclaf (Romainville, France). Genistein was from Calbiochem and ouabain from Boehringer (Mannheim, Germany). Daidzein was from LC Services Corporation (Woburn, MA); lavendustin A, compound 5 [(2-hydroxybenzyl)aminobenzoic acid], tyrphostin A$_47$ (RG-50864), methyl 2,5-dihydroxycinnamate (an analog of erbstatin) and daidzein were from LC Services Corporation; veratridine was from Sigma Chemical Co. (St Louis, MO), daidzein from Calbiochem and ouabain from Boehringer (Mannheim, Germany).

Abbreviations: BTX-B, batrachotoxinin A 20-α-benzoate; STX, saxitoxin; α-ScTx, α-scorpion toxin; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.
Cell culture. Primary cultures of rat fetal brain neurons were prepared essentially as described previously (Jover et al., 1988), except that the culture medium was Dulbecco’s modified Eagle medium (GIBCO BRL, Gaithersburg, MD) containing 5% fetal calf serum (Boehringer, Mannheim). Cultures of cerebellar granule cells were obtained as described (Grigson et al., 1993).

Sodium influx. The influx of $^{22}$Na$^+$ induced by neurotoxins was measured as described previously (Couraud et al., 1986). Cultured cells were preincubated in the presence of the indicated toxins and drugs in buffer A (5.4 mM KCl, 1.8 mM CaCl$_2$, 0.8 mM MgSO$_4$, 10 mM glucose, 25 mM HEPES, 1 mg/ml bovine serum albumin and Tris-base to adjust the pH to 7.4) containing 140 mM choline chloride and 10 mM NaCl to which were added $^{22}$Na$^+$ (0.5 μCi/assay), 5 mM ouabain, neurotoxins and drugs at the concentrations specified under “Results.” At the end of the incubation time, the medium was aspirated and the cells were rinsed three times with 140 mM choline chloride in buffer A at 4°C, dissolved in 0.1 M NaOH and the accumulated radioactivity was measured.

$[^3H]$HBTX-B binding to rat brain synaptosomes. The synaptosomal crude fraction P2 was prepared as described previously (Jover et al., 1988) and aliquots were stocked at -80°C. The standard binding medium contained 140 mM choline chloride, 5 mM KCl, 1.8 mM CaCl$_2$, 0.8 mM MgSO$_4$, 2 mg/ml bovine serum albumin and 20 mM HEPES, adjusted to pH 7.4 with 1 M Tris. Concentrated solutions of veratridine, RUS9568, genistein, lavendustin A and tyrphostin A$_{17}$ were prepared in dimethyl sulfoxide. Unless mentioned, the final concentration of dimethyl sulfoxide was less than 0.1% (v/v). Concentrated solutions of $[^3H]$HBTX-B were prepared in ethanol; in all experiments, the final concentration of ethanol was less than 0.1% (v/v). $[^3H]$HBTX-B and RUS9568 were diluted in standard binding medium containing 0.04% (v/v) Emulphor EL-620, a nonionic detergent used as an emulsifier as described by Poli et al. (1986).

Synaptosomes (200 μg) were preincubated with RUS9568 (10 μM) in the standard binding medium for 60 or 80 min at room temperature. Tyrosine kinase inhibitors were added 30 min before the beginning of incubation which was started by addition of $[^3H]$HBTX-B. Incubation was carried out at 20°C and was stopped by filtering through Whatman GF/C filters under vacuum and immediately washing three times with 4 ml of standard medium at 4°C. Radioactivity was estimated by ligand scintillation counting (1600 TR master TM 47, Scientific Solution, Foster City, CA). In most experiments, capacitance and leak currents were subtracted from active currents with use of a P/4 protocol (Bezania and Armstrong, 1977). The total capacitance of cells was 6 to 8 pF. Data acquisition and analysis were controlled by pCLAMP software (Axon Instruments, Foster City, CA), and data were fitted to the following equations with the SigmaPlot nonlinear curve fitter: Na$^+$ peak current = $a/(1 + \exp(-x/a))$ for the inactivation curve, Na$^+$ peak current = $a/(1 + \exp(-x/b))$ for the activation curve, in which $x$, $k$ and $a$ are parameters determined by multiple iterations of the algorithm.

Results

Inhibition of $^{22}$Na$^+$ influx by genistein but not by other tyrosine kinase inhibitors in cultured fetal brain neurons. The effects of several tyrosine kinase inhibitors on the influx of $^{22}$Na$^+$ induced by neurotoxins were studied in cultured rat brain neurons. The influx through voltage-sensitive Na$^+$ channels was revealed by addition of a mixture of α-ScTx, which blocks channel inactivation by binding to site 3, and veratridine which alters both activation and inactivation by binding to site 2 (Catterall, 1980). Cells were preincubated for 20 min in a Na$^+$-free medium with α-ScTx and the different tyrosine kinase inhibitors. $^{22}$Na$^+$ influx was then elicited for 30 s in the presence of a mixture of 20 nM α-ScTx, 5 μM veratridine and the inhibitors. In these conditions, genistein at 250 μM completely inhibited the toxin-induced $^{22}$Na$^+$ influx (fig. 1A). On the contrary, lavendustin A (10 μM), compound 5 (10 μM), tyrphostin A$_{17}$ (250 μM) and the erbstatin analog (10 μM) had no significant effect on neurotoxin-induced $^{22}$Na$^+$ influx. These drugs have been shown to specifically inhibit tyrosine kinase activity in rat hippocampus with IC$_{50}$ of 18 μM for genistein, 0.5 μM for lavendustin A and compound 5 and 8 μM for tyrphostin A$_{17}$ (O’Dell et al., 1991), which indicated that at concentrations used in our experiments kinase inhibition was complete. Daidzein, a genistein analog that lacks tyrosine kinase inhibitory activity (Akiyama and Ogawara, 1991), was also able to block $^{22}$Na$^+$ uptake. The dose-response curves of genistein and daidzein indicate IC$_{50}$ values for $^{22}$Na$^+$ influx of 60 μM and 195 μM, respectively (fig. 1B). In agreement with the dose-response curve, we observed a 72 ± 2% inhibition of $^{22}$Na$^+$ influx at 250 μM daidzein and a complete inhibition at 250 μM genistein (data not shown). To measure the time course of genistein action, we preincubated cultured neurons with 100 μM genistein for different periods of time before a 15-s period of $^{22}$Na$^+$ uptake. Figure 1C shows that maximum sodium flux inhibition was obtained within 20 s, which indicated that the time course of genistein interaction with intact cells was very rapid.

Inhibition of sodium current by genistein in cultured cerebellar granule cells. Voltage-sensitive sodium currents were measured in cultured cerebellar granule cells by the patch-clamp technique in the whole-cell configuration. The Na$^+$ gradient was reversed to eliminate variability in the space clamp, allowing recordings of highly reproducible peak currents (Numann et al., 1991; Dargent et al., 1994). The external solution contained 90 mM choline Cl, 5 mM Na acetate, 15 mM tetraethylammonium-Cl, 1 mM MgCl$_2$, 1.5 mM CaCl$_2$, 1 mM KCl, 5 mM glucose, 0.2 mM CdCl$_2$ and 30 mM HEPES (pH adjusted to 7.3 with tetramethylammonium-OH). The internal solution contained 100 mM NaF, 30 mM NaCl, 20 mM CsF, 5 mM HEPES (pH adjusted to 7.3 with CsOH). Tetraethylammonium and Cs were used to ensure minimal K$^+$ contribution to the outward Na$^+$ channel currents. Currents were recorded by a Biologic (Grenoble, France) RK-300, low pass filtered at 2 kHz with an eight-pole Bessel filter and sampled at 20 kHz with a 12-bit ADC (Labormaster TM 40, Scientific Solution, Foster City, CA). In most experiments, capacitance and leak currents were subtracted from active

\[ \text{Inhibition of sodium current by genistein} \]
Application of 20 μM genistein induced a significant reduction of the Na⁺ peak current amplitude (n = 6 cells). Figure 3B indicates that changes in the voltage dependency of both activation and inactivation could be detected. After treatment with 20 μM genistein, a shift to the left of the voltage-inactivation curve was observed, whereas the voltage-activation curve shifted about 20 mV toward more positive potentials. This shift was mainly caused by a change in the slope of the curve which makes the interpretation difficult.

The changes were complete 5 min after genistein was added to the cell bath medium. In the same experimental conditions, lavendustin A (10 μM) was ineffective and tyrphostin A47 (100 μM) showed no significant effect (data not shown).

Inhibition of [3H]BTX-B binding to rat brain synaptosomes by genistein. To analyze the molecular mechanism of the apparent competition between genistein and veratridine, we have looked at the effect of genistein on the binding of [3H]BTX-B to rat brain synaptosomes. Because the
level of specific binding of the latter toxin was low, experiments were done in the presence of the pyrethroid RU39568 (10 μM) that was shown to increase the affinity of [3H]BTX-B to site 2 (Lombet et al., 1988; Trainer et al., 1993). In these conditions, binding equilibrium of [3H]BTX-B to synaptosomes was obtained after 16 h at 26°C as shown in figure 5A. Dissociation experiments (fig. 5B) allowed the calculation of a dissociation rate constant $k_{-1}$ of $5.0 \times 10^{-5}$ s$^{-1}$ and with data from the association kinetics the association rate constant $k_1$ was calculated at $5.5 \times 10^{3}$ s$^{-1}$ M$^{-1}$, which gives an equilibrium dissociation constant $K_d = k_{-1}/k_1$ of 9 nM. A value of 15 nM has been obtained in similar conditions, i.e., in the presence of 10 μM RU39568 on synaptosomal membranes by Lombet et al. (1988), whereas a higher affinity ($K_d = 1.5$ nM) has been measured on solubilized and purified sodium channel (Trainer et al., 1993). The difference could be a consequence of the voltage dependence of pyrethroid interaction with sodium channels, synaptosomal membranes and frozen P2 fractions probably being depolarized compared with purified and reconstituted channels.

In the presence of 500 μM genistein, a partial decrease in the level of [3H]BTX-B bound at equilibrium (fig. 5A) was observed and was shown to be dependent on the concentration of genistein (fig. 5C). However, the effect of genistein could not be studied at higher concentration because of insolubility of the drug. Assuming that the inhibition of [3H]BTX-B binding is complete for higher concentrations of genistein, the IC$_{50}$ was 271 μM in this experiment and the mean value measured from four independent experiments was 206 μM. The inhibition could be caused by either a direct competition between genistein and BTX for the same binding site, or by an indirect negative cooperativity between the two. To clarify this point, we have looked at the effect of genistein on the dissociation kinetics of [3H]BTX-B. Figure 5B shows that addition of 500 μM genistein did not induce an increase in the [3H]BTX-B dissociation rate as would have been expected in the case of negative allosteric interaction, but, on the contrary, a small and significant decrease of the $k_{-1}$ value ($3.7 \times 10^{-5}$ s$^{-1}$).

We have examined the effects of other tyrosine kinase inhibitors on the [3H]BTX-B binding to rat brain synaptosomes (fig. 6). Lavendustin A (5 μM) and tyrphostin A$_{47}$ (100 μM) did not induce any significant change in the [3H]BTX-B binding level either in the presence or in the absence of 500 μM genistein, whereas a 73% decrease in total tyrosine kinase activity was observed (data not shown).

Finally, genistein was unable to modify the binding of [3H]STX to cultured fetal brain neurons (data not shown).
Discussion

In this paper, we have analyzed the effects of genistein, a specific tyrosine kinase inhibitor, on voltage-sensitive sodium channels. We have shown that this drug induced a blockage of $^{22}\text{Na}^+$ influx through neurotoxin-activated sodium channels in cultured brain neurons and a decrease of $\text{Na}^+$ current in cultured cerebellar granule cells. At this point, the question was whether the inhibitory effect was mediated by the inhibition of a tyrosine kinase activity. Several data argue against this hypothesis: 1) only genistein but not the other tested tyrosine kinase inhibitors was active on $^{22}\text{Na}^+$ influx or $\text{Na}^+$ current; 2) the effect of genistein was mimicked by daidzein which is described as a genistein analog inactive on tyrosine kinase activity; 3) the time course of genistein activity on intact neuronal cells was very rapid because the maximum effect was obtained in less than 20 s, which seems incompatible with an effect caused by a dephosphorylation revealed by the inhibition of a tyrosine kinase; 4) a direct interaction between genistein and sodium channels was visualized in rat brain synaptosomal fractions by the inhibition of $[^3\text{H}]\text{BTX-B}$ specific binding induced by genistein and not by other tyrosine kinase inhibitors. The two latter arguments also allow the elimination of a possible effect through the inhibition of another protein kinase, the protein histidine kinase, which has been shown to be sensitive to genistein with an IC$_{50}$ of 110 $\mu$M (Huang et al., 1992).

Regarding the site of action of genistein, it is clear that it has no effect on binding of STX to neurotoxin receptor site 1. In contrast, genistein has strong effects on veratridine and batrachotoxin action and binding at receptor site 2. This could be caused either by direct competition at this binding site or by an indirect allosteric interaction similar to what has been observed with local anesthetics and some antiarrhythmic and anticonvulsant drugs (Catterall, 1987). These drugs have been shown to accelerate the dissociation of the...
preformed batrachotoxin-receptor complex (Postma and Catterall, 1984) whereas, on the contrary, genistein induced a small decrease in the dissociation kinetics, which is not in agreement with negative cooperativity. However, like local anesthetics, genistein induced a shift in the voltage dependence of inactivation to the more negative potentials (Catterall, 1987; Ragsdale et al., 1991). Although genistein alters the voltage dependence of the rat brain Na⁺ channel, it could not be ignored that it may also decrease the channel conductance.

An alternative explanation for the activity of genistein is that the inhibition of [³H]BTX-B binding was caused by competition between genistein and RU38568 for the same binding site, inducing a decrease in the level of bound pyrethroid and thus a decrease in its stimulatory action on site 2. This hypothesis can be excluded because the effect of genistein on [³H]BTX-B binding was measured at two concentrations of RU38568, 10 μM and 50 μM, and no change in the apparent affinity of genistein was detected (data not shown).

Thus, it seems that genistein competes with BTX for the same binding site on rat brain Na⁺ channels, but we cannot exclude an allosteric effect that does not induce an increase in the dissociation kinetics of [³H]BTX-B from the preformed complex.

In conclusion, this paper mainly demonstrates that genistein, a drug very often used as a specific tyrosine kinase inhibitor, is also able to block neuronal voltage-sensitive sodium channels in a direct manner and not through the inhibition of a tyrosine kinase activity.

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References


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