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Genistein directly inhibits native and recombinant NMDA receptors

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ABSTRACT

The protein tyrosine kinase (PTK) inhibitor genistein has been widely used to examine potential effects of tyrosine phosphorylation on neurotransmitter function. We report here that genistein inhibits N-methyl-D-aspartate (NMDA) receptors through a direct effect. Whole-cell NMDA-activated current was recorded in native receptors from mouse hippocampal slice culture and rat recombinant NR1aNR2A and NR1aNR2B receptors transiently expressed in HEK293 cells. Extracellular application of genistein and NMDA reversibly inhibited NMDA-activated current. The inhibition of NMDA-activated current by genistein applied externally was not affected when genistein was also pre-equilibrated in the intracellular solution. Daidzein, an analog of genistein that does not block PTK, also inhibited NMDA-activated current. Coapplication of lavendustin A, a specific inhibitor of PTK, had no effect on the NMDA response. Moreover, genistein-induced inhibition of NMDA-activated current displayed concentration- and voltage-dependence. Our results demonstrate that genistein has a direct inhibitory effect on NMDA receptors that is not mediated via inhibition of tyrosine kinase. Thus, other PTK inhibitors may be more suitable for studying involvement of PTKs in NMDA receptor-mediated events.

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1. Introduction

Genistein (4,7,4′-trihydroxyisoflavone) is one of several known plant-derived soy isoflavones found in high abundance in most soy food products. Genistein, the most studied of the soy phytoestrogens which are structurally and functionally similar to estradiol, exerts a number of biological effects such as estrogenic (Kuiper et al., 1998), antioxidant activity (Tikkanen et al., 1998) and anticancer effects (Pan et al., 2001). Genistein is also a potent protein tyrosine kinase (PTK) inhibitor (Akiyama et al., 1987; Akiyama and Ogawara, 1991; Hanke et al., 1996) which is widely used as a pharmacological tool to assess the involvement of PTK-mediated signaling. In addition to inhibition of PTKs, genistein has been reported to directly inhibit a number of ion channels. These include voltage-gated channels selective for Ca^{2+} (Belevych et al., 2002; Kurejova and Lacinova, 2006; Tao et al., 2009; Yokoshiki et al., 1996), Na^{+} (Paillart et al., 1997), and K^{+} (Smirnov and Aaronson, 1995). Genistein has also been shown to inhibit ligand-gated ion channels, including both $GABA_A$ (Dunne et al., 1998; Huang et al., 1999) and glycine receptors (Huang and Dillon, 2000; Zhu et al., 2003). Moreover, genistein inhibits depolarization-induced Ca^{2+} influx and glutamate release from hippocampal synaptosomes, most likely through direct inhibition of voltage-gated Ca^{2+} channels (VDCC) and/or K^{+} channels (Pereira et al., 2003).

The NMDA receptor is one of three main subtypes of ionotropic glutamate receptors that mediate most fast excitatory neuronal transmission. NMDA receptors are multiprotein complexes with a central ion pore. Activation of NMDA receptors results in the opening of a non-selective cation channel, which allows flow of Na^{+} and Ca^{2+} ions into the cell. Calcium flux through NMDA receptors is thought to play a critical role in synaptic plasticity, synaptogenesis and excitotoxicity (Bliss and Collingridge, 1993; Choi, 1992; Malenka and Nicoll, 1999). A number of studies suggest that phosphorylation of the NMDA receptor is an important component in receptor function (Chen and Roche, 2007). PTKs, especially non-receptor PTKs (Src and Fyn), are important modulators of NMDA receptors (Ali and Salter, 2001). For example, NMDA currents are potentiated by increasing PTK activity and reduced by decreasing PTK activity (Wang and Salter, 1994; Wang et al., 1996). Activation of NMDA receptors is required for long-term potentiation (LTP) of excitatory synaptic transmission at hippocampal CA1 synapses. The blockade of PTK activity significantly inhibits NMDA receptor-dependent LTP (Casey et al., 2002; Huang and Hsu, 1999; O'Dell et al., 1991). Intracranial administration of PTK inhibitors impairs long-term memory formation in newborn chicks (Whitechurch et al., 1997). The involvement of PTKs in modulation of NMDA receptor-dependent processes in those studies was tested using genistein as the PTK inhibitor. The observed effect induced by genistein was interpreted as a functional consequence of inhibition of PTK activity.

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However, given the fact that genistein exerts a variety of complicated biological effects, especially effects on voltage- and ligand-gated ion channels that are PTK-independent, we assessed the ability of genistein to directly inhibit NMDA receptor function.

2. Methods

2.1. Hippocampal neurons

Organotypic explants were derived from postnatal day (P) 4 (day of birth = P1) hippocampus, obtained from pups born of timed-pregnant C57Bl/6 mice as previously described (Singh et al., 2000). The hippocampus was first dissected out from the brain, and then sliced cross-sectionally, resulting in ~360 μm sections. Explant slices were maintained as roller tube cultures (Gahwiler, 1981) on rat tail collagen-coated/poly-L-lysine pre-coated glass coverslips and were grown in steroid-deficient and phenol red-free maintenance medium [gelding serum (25%); Hank's BSS (22.5%); BME (50%); glucose (6 mg/ml); L-glutamine (2 mM); ascorbic acid (50 μg/ml)]. This maintenance medium was replaced 3 times a week.

2.2. Recombinant receptors

cDNAs encoding rat NR1a, NR2A and NR2B were generous gifts from Dr. David Lynch (University of Pennsylvania). Human embryonic kidney (HEK293) cell line was transiently transfected with recombinant NMDA receptor subunits using TransIT®-293 transfection reagent (Mirus, Madison, WT).

Briefly, HEK293 cells were washed and placed in fresh Dulbecco's modified eagle medium containing 10% FBS and antibiotics (penicillin 100 U/mL). NR1a along with NR2A or NR2B (0.5:0.5 μg) was added to cells growing exponentially on one poly-L-lysine coated coverslip placed in a 35-mm culture dish. Transfected cells were used for electrophysiological analysis 24–48 h after the transfection.

2.3. Electrophysiology

Whole-cell patch recordings were made at room temperature (22–25 °C) at a holding potential of −60 mV. Patch pipettes of borosilicate glass (M1B150F, World Precision Instruments, Inc., Sarasota, FL) were pulled (Flaming/Brown, P-87/PC, Sutter Instrument Co., Novato, CA) to a tip resistance of 7–8 MΩ. The pipette solution contained (in mM): 140 CsCl, 10 EGTA, 10 HEPES, 4 Mg–ATP; 0.2 Na_3–GTP, pH 7.2. The slice or transfected cells on coverslip was superfused (7–10 ml/min) with extracellular solution containing (in mM): 125 NaCl, 5.5 KCl, 5.0 $CaCl_2$, 20 HEPES, 10 D-glucose, 10 μM glycine; pH 7.3. For brain slice recordings, 100 nM tetrodotoxin (TTX) and 5 μM bicuculline methiodide were added to the extracellular solution to suppress spontaneous activity. The cells were visualized using an upright, fixed stage microscope (Nikon Optiphot-2UD) equipped with standard Hoffman modulation contrast (HMC) optics and a video camera system (Sony model XC-75 CCD video camera module, DOT-X monitor). NMDA was prepared in extracellular solution and was applied (10 s in most cases) to cells via gravity flow using a Y-shaped tube positioned near the target cell. With this system, the 10–90% rise time of the junction potential at the open tip was 60–120 ms. NMDA-evoked currents from the whole-cell configuration were obtained using a patch clamp amplifier (Axopatch 200A, Axon Instruments, Foster City, CA) equipped with a CV201A headstage. The currents were low-pass filtered at 5 kHz, monitored on an oscilloscope and a chart recorder (Gould TA240), and stored on a computer (pClamp 6.05, Axon Instruments) for subsequent analysis. 60–80% series resistance compensation was applied at the amplifier. To monitor the possibility that access resistance changed over time or during different experimental conditions, at the initiation of each recording we measured and stored on our digital oscilloscope the current response to a 5 mV voltage pulse. This stored trace was continually referenced throughout the recording. If a change in access resistance was observed throughout the recording period, the patch was aborted and the data were not included in the analysis.

2.4. Chemicals

NMDA, MK-801, bicuculline methiodide and glycine were purchased from Sigma. TTX was obtained from Tocris (Ellisville, MO) and genistein and daidzein from either Sigma or LC Laboratories (Woburn, MA). NMDA, glycine, TTX and MK-801 stocks were made in double distilled H_2O. Bicuculline, genistein and daidzein were prepared in dimethyl sulfoxide (DMSO). The final concentration of DMSO was less than 0.05% (v/v).

2.5. Data analysis

Peak currents were normalized to the initial response (100%). NMDA concentration-response profiles were fitted to the following equation: $I/I_{max} = 1(1+(EC_{50}/[NMDA]^n))$, where I and I_{max} represent the normalized NMDA-activated current at a given concentration and the maximum current induced by saturating a [NMDA], EC_{50} is the half-maximal effective NMDA concentration, and n is the slope factor.

All data were presented as means ± SEM. Student's t-test (paired or unpaired) or one-way ANOVA with Student–Newman–Keuls multiple comparison test was used to determine statistical significance ($p < 0.05$).

3. Results

3.1. Inhibition of NMDA-activated current by genistein and daidzein

The average amplitude of whole-cell current activated by 50 μM NMDA in cultured hippocampus neurons (days in culture, 8–16) was 623 ± 87 pA ($n = 38$). The inward NMDA-activated current was completely blocked by MK-801 (10 μM), a selective NMDA receptor inhibitor (data not shown). As shown in Fig. 1, coapplication of genistein with 50 μM NMDA resulted in a concentration-dependent decrease in NMDA-activated current. 50 and 100 μM genistein inhibited mean amplitude of NMDA currents to 77 ± 3.9% and 70 ± 2.7% of the control, respectively ($p < 0.05$, one-way ANOVA, $n = 4–6$). Genistein mainly inhibited the current amplitude without apparently affecting steady-state current (Fig. 1A). In addition, the NMDA current fully recovered to the control level after 1–3 min of washout (Fig. 1A). To examine whether the observed effect was due to the inhibition of PTKs, daidzein (4′,7-dihydroxyisoflavone), a genistein analog which does not inhibit PTKs, was coapplied with

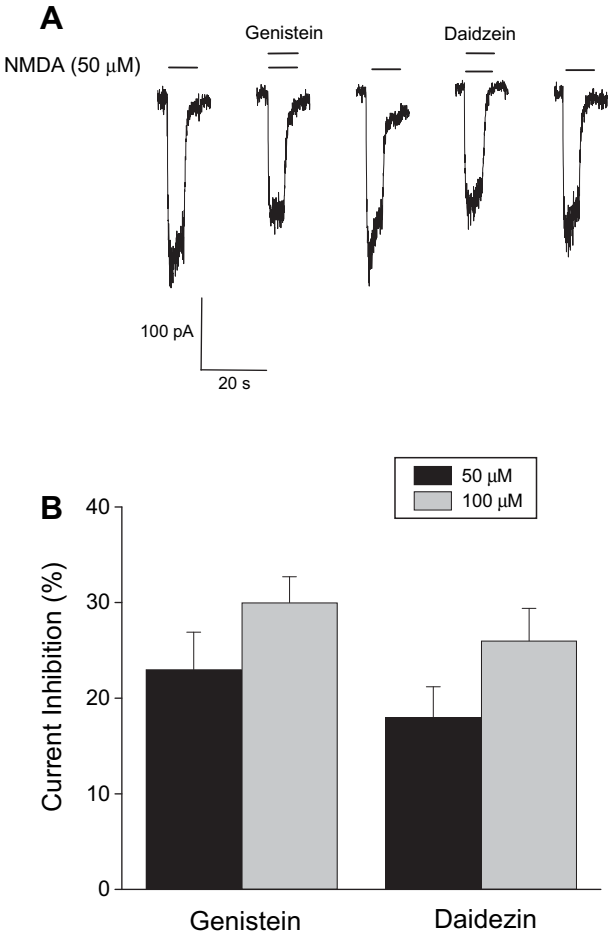

Fig. 1. Effect of coapplied genistein or daidzein on NMDA-activated currents. A, Whole-cell NMDA (50 μM)-activated current was recorded from one hippocampal neuron. Genistein (50 or 100 μM) or daidzein (50 or 100 μM) was coapplied with NMDA to the cell for 10 s. Note that genistein- and daidzein-induced inhibition of NMDA current was reversible. B, Summary data of inhibition of NMDA response by genistein or daidzein. The current amplitude is normalized to the initial NMDA response (assigned as 100%). Each data point represents Mean ± SEM from at least 4 cells from slices at P08–P15.

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