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Non-competitive Inhibition of Kainate-Induced Currents by Diethylstilbestrol in Acutely Isolated Mouse CA1 Hippocampal Neurons

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ABSTRACT—The effect of a synthetic estrogen, diethylstilbestrol (DES), on kainate-induced currents was investigated in the hippocampal CA1 pyramidal neurons acutely dissociated from the mice using the nystatin-perforated patch-clamp recording configuration under voltage-clamp conditions. DES inhibited the current evoked by 100 μM kainate in a concentration-dependent manner with a half-maximum inhibitory concentration of 8.8 μM. The action of DES was voltage-independent. Since DES produced a suppression of the maximum response of the kainate concentration-response curve, the inhibition by DES of the kainate-induced current appears to be non-competitive.

Keywords: Nystatin-perforated patch-clamp recording, Diethylstilbestrol, Environmental endocrine disrupter

A number of human-made chemicals with the potential to disrupt the endocrine system in wildlife and humans have been released into the environment. They are called environmental endocrine disrupters, and some of these chemicals are known to have estrogen-like activities (1). The exposure to these environmental estrogens during the prenatal and neonatal period is thought to be able to influence the central nervous system (CNS), because gonadal steroid hormones have profound effects on the developmental organization of the nervous system (2). Although the historical view of steroid action focuses on the genomic effects via intracellular steroid receptors, it is now well known that many of the steroids can modulate the excitability of CNS neurons through the interaction with ligand-gated and voltage-dependent ion channels (3 – 6).

In hippocampal neurons, the excitatory synaptic transmission has been thought to be mostly mediated by glutamate acting on postsynaptic glutamate receptors including α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate and *N*-methyl-D-aspartate (NMDA) receptors. The AMPA- and kainate-receptor subtypes have frequently been referred as 'non-NMDA' receptors because the identification of responses mediated by native kainate receptors is made difficult by the agonist action of kainate at AMPA receptors (7). The effects of steroids on the NMDA receptor have been extensively studied (4, 5). In contrast, relatively little is known about the action of steroid on the non-NMDA receptors. Recently, the AMPA- and kainate-induced currents have been reported to be inhibited by the neurosteroid pregnenolone sulfate (8). However, since non-sulfated pregnenolone has no effect on the kainate response (6), it can be speculated that the sulfation plays a crucial role in the mechanism underlying inhibitory action of steroids on kainate-induced currents. Thus, it is of interest to investigate whether or not the endocrine disrupter without sulfate moiety affects the kainate response. For this purpose, we observed the effect of the synthetic estrogen diethylstilbestrol (DES), which is known as a potent endocrine disrupter in rodents (9), on the kainate-induced currents in the pyramidal neurons acutely dissociated from the hippocampal CA1 region of neonatal mice using the nystatin-perforated patch-clamp technique (10).

The hippocampal CA1 pyramidal neurons were freshly dissociated from immature (10- to 14-day-old) ddY mice anesthetized with pentobarbital sodium (50 mg/kg, i.p.) as previously described (11). In brief, the brain was quickly removed and cut into 300-μm-thick coronal slices. The slices were incubated in the normal external solution containing pronase (0.1 mg/ml) for 40 min, followed by thermolysin (0.1 mg/ml) for 20 min at 31°C. Thereafter, the CA1 region of hippocampus was punched out and transferred to a culture dish filled with external solution. Finally, neurons were mechanically dissociated with a fire-polished micropipette. The composition of the normal external solution was: 150 mM NaCl, 5 mM KCl, 1 mM

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$MgCl_2$, 2 mM $CaCl_2$, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) and 10 mM glucose. The composition of the patch-pipette solution was 40 mM CsCl, 110 mM Cs-methanesulfonate, 10 mM HEPES and 0.2 mM nystatin. The pH of the normal external and patch-pipette solutions was adjusted to 7.4 and 7.2, respectively, with tris(hydroxymethyl)aminomethane (Tris-base). The membrane current was measured with a patch-clamp amplifier (Axopatch-1D; Axon Instruments, Foster City, CA, USA) by the use of the nystatin-perforated patch-clamp technique at room temperature (21 – 24°C). Unless otherwise specified, a holding potential (V_H) of −40 mV was employed throughout the experiment. Currents were filtered at 1 kHz using an eight-pole Bessel filter (No. 3611; NF Electronic Instruments, Tokyo), monitored by an oscilloscope (CS-6040; Kenwood, Tokyo) and a pen recorder (RTA-1200; Nihon-Kohden, Tokyo). Drugs were applied by the Y-tube method that allows the complete exchange of external solution surrounding the recording neuron within 20 ms (11). Data were analyzed by Student's paired *t*-test. *P* values of less than 0.05 were considered significant. All drugs were obtained from Sigma (St. Louis, MO, USA). A stock solution of DES was prepared in dimethyl sulfoxide (DMSO), and the final concentration of DMSO was <0.3%, at which DMSO alone had no effect on the kainate-induced current.

The application of kainate evoked the inward currents. In agreement with a previous report (6), the kainate-induced current showed little or no desensitization and did not decline with repeated application. Figure 1 shows the concentration-dependent effect of DES on the current induced by 100 μM kainate. DES at a concentration as low as 1 μM slightly but significantly inhibited the kainate response (Fig. 1: A and B). In order to quantitatively estimate the potency of DES, the concentration-response curve for inhibition of the 100 μM kainate response by DES was constructed. As shown in Fig. 1B, curve fit analysis revealed an IC_{50} of 8.8 μM. Furthermore, we investigated the effect of 10 μM DES on the current evoked by 100 μM kainate at various V_Hs. The reversal potential of the kainate-induced currents were 3.8 ± 0.2 mV (n = 4) and 3.5 ± 0.4 mV (n = 4) without and with DES, respectively. DES (10 μM) inhibited the kainate-induced current by about 55% at each holding potential tested (Fig. 1C). There was no significant difference among the inhibition ratios at various V_Hs, indicating that the action of DES may be voltage-independent.

The effect of 10 μM DES on the concentration-response curve of kainate-induced currents was investigated. In this experiment, all responses were normalized to the current induced by 100 μM kainate alone. As shown in Fig. 2A, DES significantly inhibited the maximum response, thus suggesting that the action of DES may be non-competitive.

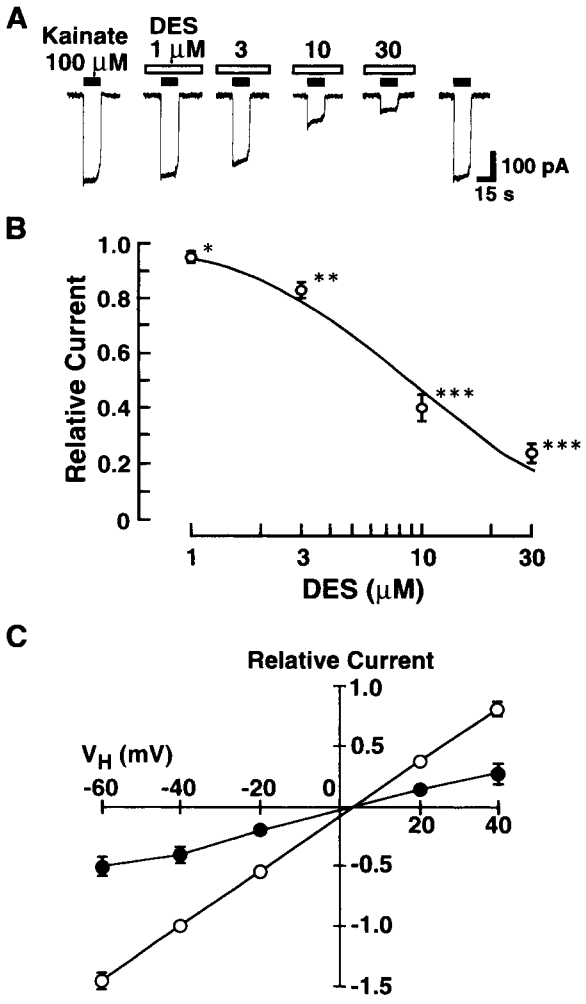

Fig. 1. Inhibition of the kainate-induced current by DES. A: Consecutive current traces showing the kainate (100 μM)-induced currents in the absence and presence of various concentrations of DES. Holding potential (V_H) was −40 mV. Kainiate was applied for 12 s every 3 min. DES was applied for 1 min from 20 s before the application of kainate. B: Concentration-inhibition curve for DES on 100 μM kainate-evoked currents. Currents were expressed as the relative value to the control response induced by 100 μM kainate alone. Each point is the average of 4 – 5 experiments. Vertical bars indicate ± S.E.M. The curve shows the least-squares fit to the equation $I = 1 - C^n / (C^n + K^n)$, where I is relative current amplitude, C is DES concentration, n is Hill coefficient (1.2) and K is the half inhibition concentration. *$P<0.05$, **$P<0.01$, ***$P<0.001$ (vs response to kainate alone). C: Current-voltage relationships for 100 μM kainate in the absence (open circle) and presence (filled circle) of 10 μM DES. Current amplitudes were normalized to that at −40 mV without DES. Each point is the average of 4 experiments which was obtained from 4 neurons. Vertical bars indicate ± S.E.M.

The half-maximum effective concentration and maximum response of kainate were 68.5 μM and 1.62 in the absence and 78.2 μM and 0.68 in the presence of DES (10 μM), respectively. The Lineweaver-Burk plot also confirms a non-competitive mode of the inhibitory action (Fig. 2B).

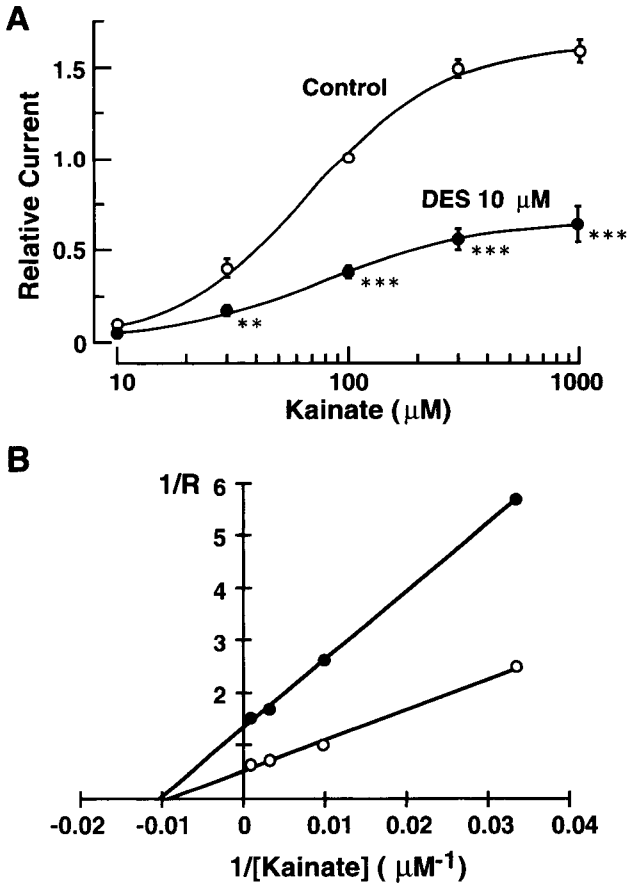

Fig. 2. Characterization of the inhibition by DES on the kainate response. A: Concentration-response curves for kainate without (open circle) or with (filled circle) 10 μM DES. All responses were normalized for the current induced by 100 μM kainate alone. Each point is the average of 4–5 data, and total neuron number was 5. Vertical bars show ± S.E.M. The curve shows the least-squares fit to the equation $I = I_{max} \cdot C^n / (C^n + K^n)$, where I_{max} is maximum response, C is the concentration of the agonist, n is Hill coefficient (1.3 and 1.2 in the absence and presence of DES, respectively) and K is the half-maximum activation. $^{**}P<0.01$, $^{***}P<0.001$ (vs control). B: Lineweaver-Burk plot of the concentration-response relationships.

In the present paper, we demonstrated that the synthetic estrogen DES has a potent, concentration-dependent and non-competitive inhibitory action on the kainate-induced current in immature mouse hippocampal CA1 pyramidal neurons. As mentioned above, sulfation is thought to play a crucial role in the mechanism by which steroids inhibit the kainate-induced currents (5). However, the present result suggests that DES also has the inhibitory action on the kainate-response even though it does not have a sulfate moiety.

DES is known as a potent prenatal and neonatal endocrine disrupter in the rodents (9). On the other hand, DES can be converted from diethylstilbestrol diphosphate (fosfestrol), which is clinically used in the therapy for prostatic carcinoma (12, 13). However, at present, there is no information about the distribution of DES converted from fosfestrol in the brain. Thus, further studies are needed to clarify whether fosfestrol and its metabolites have inhibitory action on the kainate response.

Exogenous steroid hormones are thought to activate appropriate nuclear receptors that regulate the expression of target gene sequences, and the alpha and beta forms of the estrogen receptor have been cloned as a member of the nuclear receptor superfamily (14). Such nuclear effects are usually detectable on the time scale of hours or longer following hormone administration. On the other hand, steroid hormones have been reported to produce a rapid non-genomic response through plasma membrane binding sites (14). In the present study, the concentration of DES required to block the current was several orders higher than that required for the gene expression (14, 15). Furthermore, the rapid onset of the action of DES and its relatively rapid reversibility are not consistent with a response requiring gene transcription. Therefore, the DES-induced inhibition of the kainate response may be due to a non-genomic mechanism. However, since the non-genomic mechanism of estrogen action remains largely unknown, a full understanding of the inhibitory mechanism of DES on the kainate response will require further investigation.

To date, little is known about the activity of environmental endocrine disrupters in the mammalian brain. By demonstrating an inhibitory effect of DES on kainate-induced currents, the present study suggests that DES might modify functional properties of neurons expressing the non-NMDA receptors. Although we must further characterize the pharmacological, toxicological and pathophysiological actions of DES and other endocrine disrupters, we are beginning to develop our understandings of how these chemicals actually work in the brain.

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