The ES-242s, Novel N-Methyl-d-aspartate Antagonists of Microbial Origin, Interact with Both the Neurotransmitter Recognition Site and the Ion Channel Domain*

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ES-242-1-5 are novel microbial bioxanthracenes which do not contain nitrogen. The ES-242s inhibited the binding of [3H]TCP and [3H]CGS19755 to the N-methyl-d-aspartate (NMDA) receptor complex. They had no effect on the binding of the specific ligands for the non-NMDA receptor. The biochemical and pharmacological properties of ES-242-1 were fully examined since it is the most potent of the five compounds. ES-242-1 is highly specific for the NMDA receptor; it has no effect on other receptors. Kinetic analyses indicated that ES-242-1 inhibited the binding of [3H]TCP and [3H]CGS19755 in a competitive manner, respectively, suggesting that ES-242-1 interacts with both the transmitter recognition site and the channel domain. ES-242-1 selectively inhibited NMDA-induced Ca2+ influx in primary cultures of mouse hippocampal neurons. ES-242-1 also specifically blocked the increase in cyclic GMP accumulation induced by NMDA or L-glutamate in rat cerebellar slices. In a concentration range of 0.1-1.0 μM, ES-242-1 was as potent as MK-801 in preventing glutamate-induced neurotoxicity in primary cultures of mouse hippocampal neurons. These results show that ES-242-1 is a potent and specific antagonist for the NMDA receptor. The antagonistic properties of the ES-242s appear to be due to a novel mechanism of action at the NMDA receptor.

The excitatory amino acids L-glutamate and L-aspartate are thought to act as principal neurotransmitters in the central nervous system (1). The receptors that mediate their actions are divided into three major subtypes, N-methyl-d-aspartate (NMDA),1 quisqualate/kainate (non-NMDA), and metabotropic, based on their sensitivity to specific agonists and their physiological properties. The NMDA receptor is well characterized and plays an essential role in the induction of use-dependent modifications of synaptic efficiency (2, 3) such as long term potentiation in the CA1 region of the hippocampus that is thought to be a key electrophysiological event in memory formation.

In spite of their physiological importance, excitatory amino acid receptors can also be responsible for neuronal injury. Although the exact mechanism of cell injury is still uncertain, the brain damage associated with anoxia, stroke, hypoglycemia, and other neurodegenerative diseases may be at least partially produced by excessive activation of the NMDA receptor. It is thought that depolarization, influx of several kinds of ions, water entry into cells, activation of proteases and lipases by an increase of intracellular Ca2+ concentration, and mitochondrial dysfunction caused by excessive stimulation of the NMDA receptor may lead to the subsequent cell death (4, 5). Therefore, it has been proposed that the pharmacological blockade of the NMDA receptor-mediated neurotoxicity may be an effective approach to the treatment of brain injury.

The NMDA type of excitatory amino acid receptor has been shown to possess a number of distinct sites through which its function may be regulated or pharmacologically modified (1). These include: (i) the agonist recognition site, at which glutamate, aspartate, and NMDA bind to open the NMDA receptor channel. The glutamate analogues APV (2-amino-5-phosphonovaleric acid) (6), CGS19755 (7), and CPP (8) are

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1The abbreviations used are: NMDA, N-methyl-d-aspartate; TCP, thienylcyclohexylpiperidine; CSM, crude synaptic membranes; CPP, 3-(2-carboxypiperazine-4-yl)propyl-1-phosphonic acid; AMPA, (R,S)-a-amino-hydroxy-5-methyl-isoxazole-4-propionic acid; 3-PPP, propyl-3-[3-hydroxyphenyl]piperidine; [Ca2+]i, intracellular free Ca2+ concentration; (NO2)4Arg, L-Nω-nitroarginine; NO, nitric oxide; EGTDA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; MOPS, 4-morpholinepropanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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Fig. 1. The chemical structures of ES-242s.
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The displacement curves of ES-242s against [3H]TCP (A) or [3H]MK-801 (B and C) binding to rat crude synaptic membranes. Binding studies were carried out as described under "Experimental Procedures." ES-242s were dissolved in methanol at various concentrations and 10 μl was added to the reaction mixture. A, specific binding of [3H]TCP was measured in the presence of varied amount of ES-242-1 (Ο), ES-242-2 (★), ES-242-3 (●), ES-242-4 (■), ES-242-5 (△), and MK-801 (+). B, specific binding of [3H]MK-801 was measured in the presence of varied amount of ES-242-1 (Ο), ES-242-2 (★), ES-242-4 (■), ES-242-5 (△), and MK-801 (+). C, the displacement curve of ES-242-1 (Ο) or CPP (△) against [3H]MK-801 binding in the presence or absence of 100 μM L-glutamate. Open symbols indicate the values in the presence of L-glutamate and closed symbols in the absence of L-glutamate. All the experiments were performed in duplicate.

**TABLE I**

IC_{50} values of various ES-242 compounds for binding of different ligands

Data represent means ± S.E. of more than three independent experiments in duplicate.

<table>
<thead>
<tr>
<th>Radioligands</th>
<th>Diacetyl-ES-242-1</th>
<th>Diacetyl-ES-242-2</th>
<th>Diacetyl-ES-242-3</th>
<th>IC_{50} values</th>
<th>μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>[3H]TCP</td>
<td>0.116 ± 0.025</td>
<td>2.9 ± 0.13</td>
<td>~2.9</td>
<td>25.3 ± 2.9</td>
<td>&gt;15</td>
</tr>
<tr>
<td>[3H]MK-801</td>
<td>0.066 ± 0.017</td>
<td>3.1 ± 0.79</td>
<td>NT</td>
<td>18.0 ± 6.7</td>
<td>NT</td>
</tr>
<tr>
<td>[3H]CPP</td>
<td>3.9 ± 0.15</td>
<td>14.5 ± 6.6</td>
<td>NT</td>
<td>10.1 ± 2.8</td>
<td>&gt;15</td>
</tr>
<tr>
<td>[3H]CGS19755</td>
<td>1.1 ± 0.39</td>
<td>15.1 ± 5.2</td>
<td>NT</td>
<td>8.7 ± 2.1</td>
<td>NT</td>
</tr>
<tr>
<td>[3H]Glycine</td>
<td>&gt;15</td>
<td>&gt;15</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
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<td>[3H]Kainate</td>
<td>&gt;15</td>
<td>&gt;15</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>[3H]AMPA</td>
<td>&gt;15</td>
<td>&gt;15</td>
<td>NT</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>[3H]PPP</td>
<td>&gt;15</td>
<td>&gt;15</td>
<td>NT</td>
<td>NT</td>
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</tr>
</tbody>
</table>

* NT, not tested.
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Competitive antagonists acting on the same site; (ii) an allosteric site, at which glycine regulates agonist-induced channel opening (9), and at which 7-Ch-kynurenic acid (10) and HA-966 (3-amino-1-hydroxy-pyrrolidine-2) (11) displace glycine binding competitively, and produce noncompetitive antagonism of receptors to NMDA; and (iii) sites on the receptor-associated ion channel for Mg2+ and for drugs (12) such as MK-801 (13), ketamine (14), TCP (15), and diarylguanidine derivatives (16). Recent observations suggest that there may be additional sites on the NMDA receptor where Zn2+ (17, 18), polyamines (19), and tricyclic antidepressants (20, 21) act to modulate its properties.

Clinical studies, however, are limited because of the small number of suitable NMDA antagonists available. During the course of screening to obtain NMDA antagonists of microbial origin, we found only one culture broth of a fungus Verticillium sp. SPC-15888 that showed a potent inhibitory activity against the binding of [3H]TCP or [3H]MK-801 to the NMDA receptor complex in crude rat brain synaptic membrane (CSM) fractions. In a previous paper, we reported fermentation, isolation, and characterization of ES-242-1 from the culture broth of the fungus (22). After that, other minor components (ES-242-2-5) having chemical structures similar to that of ES-242-1 were also isolated from the same culture broth (23). Since the ES-242s do not have nitrogen in their chemical structures, unlike the other NMDA receptor ligands reported so far, it was of interest to know how it interacts with NMDA receptors, and the mechanism by which it expresses its antagonistic activity.

EXPERIMENTAL PROCEDURES

Materials—ES-242-1-5 were purified from the culture broth of Verticillium sp. as reported (22, 23). All radioligands used for receptor binding experiments were purchased from Du Pont-New England Nuclear. MK-801 and ketamine were chemically synthesized by Dr. H. Obase and H. Kat0 in the Pharmaceutical Research Laboratories of our company. L-Glutamate was from Wako Pure Chemical Industries Ltd. NMDA and kainate were obtained from Sigma. Cyclic GMP radioimmunoassay kits were purchased from Yamasa. Reagents used in the measurement of proteins by the method of Bradford (24) were from Pierce Chemical Co. All other chemicals were of analytical grade.

Receptor Binding Assay—CSM were prepared from the brain of Wistar rats by the method of Murphy et al. (25) without detergent treatment. [3H]MK-801 or [3H]TCP binding assay was performed according to the method of Vignon et al. (15). The reaction mixtures (1 ml) containing 8.8 nM [3H]MK-801 or 2.6 nM [3H]TCP, 5 mM

FIG. 3. Kinetic analyses of the inhibition by ES-242-1 of [3H]TCP (A) and [3H]MK-801 (B) binding to rat crude synaptic membrane. Binding studies were carried out as described under "Experimental Procedures." ES-242-1 was dissolved in methanol at various concentrations, and 10 µl was added to the reaction mixture. A, Scatchard analysis of [3H]TCP binding in the absence (○) or presence of 82.7 nM (▲) or 332 nM (▲) ES-242-1. B, Scatchard analysis of [3H]MK-801 binding in the absence (○) or presence of 82.7 nM (▲) or 166 nM (▲) ES-242-1. All the experiments were performed in duplicate.

FIG. 4. The displacement curves of ES-242s against [3H]CGS19755 (A) or [3H]CPP (B) binding to rat crude synaptic membranes. Binding studies were carried out as described under "Experimental Procedures." ES-242s were dissolved in methanol at various concentrations, and 10 µl was added to the reaction mixture. A, [3H]CGS19755 binding was measured in the presence of varied amounts of ES-242-1 (○), ES-242-4 (▲), or ES-242-5 (▲). B, [3H]MK-801 binding was measured in the presence of varied amounts of ES-242-1 (○), ES-242-2 (▲), ES-242-4 (▲), or ES-242-5 (▲). All the experiments were performed in duplicate.
Tris-HCl buffer (pH 7.4), 10 μM L-glutamate, 150–250 μg of CSM, and varied concentrations of drugs, were incubated at room temperature for 30 min, then filtered through Whatman GF/B glass filters presoaked with 0.05% polyethyleneimine. The glass filters were then washed three times with 5 mM Tris-HCl (pH 7.4) by using Brandel M-24R cell harvester. The washed filters were dried, and the radioactivity on the filters was estimated by liquid scintillation counting in vials containing 3 ml of scintillation mixture ( Omnifluor, Du Pont). Non-specific binding was measured in the presence of 50 μM of unlabeled MK-801. The binding assay using [3H]CGS19755 was carried out as described by Murphy et al. (7). CSM used for [3H]CGS19755 binding were frozen and thawed three times. They were then suspended in 0.08% Triton X-100 and washed three times by centrifugation. The detergent-treated CSM were incubated with 9.5 nM [3H]CGS19755 for 15 min at room temperature. The reaction was stopped by rapid filtration, and the radioactivity was measured as described above. Non-specific binding was evaluated in the presence of 1 mM free ligands. The pellets were rinsed immediately after the centrifugation with 1-ml portions of ice-cold 50 mM Tris-HCl buffer (pH 7.4), and scintillant (Cleargel 1, Nakalai Tesque) was added. The radioactivity bound to CSM was determined by scintillation counting. Non-specific binding was measured in the presence of 1 mM L-glutamate. The binding assays with [3H]kainate and [3H]RS-a-amino-hydroxy-5-methyl-isoxazole-4-propionic acid ([3H]AMPA) were performed by means of the filtration method described by London and Coyle (26) and Murphy et al. (36). Reaction mixtures containing 100–300 μg of CSM, frozen and thawed three times as described above, and 20 nM [3H]glycine in 1 ml were incubated on ice for 30 min and then centrifuged. Non-specific binding was measured in the presence of 1 mM glycine. The binding assay using [3H]propranolol (3-5-hydroxyphenyl)piperidine ([3H]PPP) was carried out by means of filtration as described above. In brief, reaction mixtures contained 2 nM, [3H]PPP and 1 mg of untreated CSM. Reaction mixtures in 1 ml of 50 mM Tris-HCl buffer (pH 7.4) were incubated at room temperature for 45 min. The reactions were terminated by filtration through glass filters as described above. Unlabeled haloperidol (10 μM) was added for the determining non-specific binding. [3H]CHA, [3H]NECA, [3H]BW4101, [3H]clonidine, [3H]SCH23390, [3H]pyrilamine, [3H]tiotidine, [3H]QNB, [3H]OH-DPAT, and [3H]quipazine binding to adenosine A1, A2, dopamine D1, D2, histamine H1, H2, acetylcholine M1, and serotonin 5-HT1A, 5-HT2A, respectively, were measured according to methods described previously (28–36). Proteins were measured by the method of Bradford (24) using bovine serum albumin as a standard.

**Primary Culture of Mouse Embryonic Hippocampal Neurons—** Hippocampi were dissected from the brains of embryonic mice. The cells were dispersed with 0.25% trypsin and 0.01% DNase I digestion and mounted on 35-mm dishes coated with poly-L-lysine as described (37). Hippocampal granule cells were cultured in Pit medium (38, 39) containing 5% horse serum and 5% fetal calf serum, but without Ham’s F-12, at 37 °C in a humidified atmosphere of 95% air and 5% CO2. After 1 or 2 days in culture, 10 μM cytosine arabinoside was added and the cells incubated for 10 more days. For measurement of the intracellular Ca2+ concentration, the cultured hippocampal neurons were maintained with serum-free medium (40) containing Dulbecco’s modified Eagle’s medium (Nissui; containing no L-glutamate) supplemented with 1 mg/ml bovine serum albumin, 0.1 μM 1-thyroxine, 0.1 mg/ml transferrin, 1 μg/ml aprotinin, 30 nM selenium, 0.1 mg/ml streptomycin, and 100 units/ml penicillin on a poly-L-lysine-coated glass coverslip with a silicon rubber wall.

**Measurement of Intracellular Ca2+ Concentration—** The 12–14-day hippocampal neuron cultures were used. The culture medium was replaced with a basal salt solution containing 130 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 5.5 mM glucose, 20 mM HEPES-NaOH (pH was adjusted to 7.3), and 10% of the cells for 60 min at 37 °C. Neurons were superfused continuously with the basal salt solution, and drugs were added to the perfusion medium at 32–33 °C. The time course of changes in fura-2 fluorescence was measured using excitation wavelengths of 340 and 380 nm. The data were analyzed with an Argus 200 system (Hamamatsu Photonics). The ratio of fura-2/fluorescence was used as the indicator of Ca2+ concentration.

<table>
<thead>
<tr>
<th>Receptors</th>
<th>Subtypes</th>
<th>Radioligands</th>
<th>Receptor source</th>
<th>EC50 values</th>
<th>Methods described by</th>
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<tbody>
<tr>
<td>Glutamate</td>
<td>NM NMDA</td>
<td>[3H]CGS19755</td>
<td>Rat cerebrum</td>
<td>1.1 ± 0.39</td>
<td>Murphy et al. (8)</td>
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<tr>
<td>Adenosine</td>
<td>A1</td>
<td>[3H]CHA</td>
<td>Guinea pig forebrain</td>
<td>&gt;100</td>
<td>Bruna et al. (29)</td>
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<td></td>
<td>A2</td>
<td>[3H]NECA</td>
<td>Rat striatum</td>
<td>&gt;100</td>
<td>Bruna et al. (29)</td>
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<tr>
<td>Adrenaline</td>
<td>α1</td>
<td>[3H]WB4101</td>
<td>Rat forebrain</td>
<td>&gt;10</td>
<td>Greenberg et al. (30)</td>
</tr>
<tr>
<td></td>
<td>α2</td>
<td>[3H]Clonidine</td>
<td>Rat cerebral cortex</td>
<td>&gt;10</td>
<td>Greenberg et al. (30)</td>
</tr>
<tr>
<td>Dopamine</td>
<td>D1</td>
<td>[3H]SCH23390</td>
<td>Rat striatum</td>
<td>&gt;10</td>
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<td>Histamine</td>
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<td></td>
<td>H2</td>
<td>[3H]Ticlidine</td>
<td>Guinea pig cerebral cortex</td>
<td>&gt;100</td>
<td>Gottschalk et al. (33)</td>
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<tr>
<td>Acetylcholine</td>
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<td>[3H]QNB</td>
<td>Rat cerebral cortex</td>
<td>&gt;100</td>
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<td>Serotonin</td>
<td>5-HT1A</td>
<td>[3H]OH-DPAT</td>
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<td>&gt;100</td>
<td>Gozlan et al. (35)</td>
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<td>5-HT3</td>
<td>[3H]quipazine</td>
<td>NG108-15 cell</td>
<td>&gt;100</td>
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<td>PCP/α</td>
<td>[3H]3-PPP</td>
<td>Rat cerebrum</td>
<td>&gt;15</td>
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</table>
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RESULTS

Chemical Structures of ES-242s—ES-242-1 is the first microbial compound found to act on the channel domain of the NMDA receptor. ES-242-2–5, which are ES-242-1-related compounds having similar structures, were also isolated from the culture broth of Verticillium sp. SPC15898 (Fig. 1). The cardinal structural feature of ES-242s as NMDA receptor ligands is that they do not have nitrogen; all other NMDA antagonists reported so far do.

Eeffects of ES-242 Compounds on the Binding of Ligands Which Act at the Glutamate Recognition Site or the Ion Channel Domain of the NMDA Receptor—Binding experiments using [3H]TCP or [3H]MK-801, both of which are noncompetitive antagonists for the NMDA receptor, were carried out in the presence of 10 μM L-glutamate to maximally stimulate the [3H]TCP or [3H]MK-801 binding. ES-242-1, -2, -3, -4, and -5 inhibited [3H]TCP binding to rat CSM (Fig. 2A) with an ICso value of 116 ± 25, 2,900 ± 130, 2,900, 25,300 ± 2,910, and 1,000 ± 170 nM (mean ± S.E.), respectively. All ES-242 compounds also inhibited [3H]MK-801 binding in a similar concentration range (Fig. 2B). These results are summarized in Table I. Fig. 3 shows the Scatchard analyses of ES-242-1–induced inhibition of [3H]TCP and [3H]MK-801 binding. These data are compatible with the interpretation that ES-242-1 inhibited the binding of the two ligands in a competitive manner; inhibition constants were calculated to be approximately 35 and 337 nM, respectively. Since it has been shown that the antagonist CPP inhibits [3H]MK-801 binding to CSM in a competitive manner at the glutamate-binding site, we next examined the effects of ES-242-1 on the [3H]MK-801 binding in the presence or absence of a higher concentration of L-glutamate. The displacement curve of [3H]MK-801 binding by ES-242-1 did not change upon the addition of 100 μM L-glutamate. On the other hand, the binding curve of CPP, which interacts at the glutamate-binding site, was shifted to the right in the presence of 100 μM L-glutamate (Fig. 2C).

The ES-242 compounds also blocked the glutamate-binding site on the NMDA receptor. ES-242-1 was the most potent of the ES-242s in displacing [3H]CGS19755 binding to rat CSM (Fig. 4A) with an IC50 value of 1.06 ± 0.4 μM (mean ± S.E.), which is a 10-fold higher concentration than that required for displacing [3H]TCP. ES-242-2 and -5 also inhibited [3H]CGS19755 binding with IC50 values of 15.1 ± 5.2 and 8.7 ± 2.1 μM, respectively. Kinetic analyses showed that ES-242-1 inhibited the [3H]CGS19755 binding in a competitive manner (Fig. 5). Although ES-242-1, -2, and -5 also inhibited [3H]CPP binding (Fig. 4B), the inhibitory mechanisms were not definitively determined. Additionally, there is a positive correlation between the potencies of ES-242-1, -2, and -5 as inhibitors of [3H]CGS19755 binding and as inhibitors of [3H]CPP binding (r = 0.97) or [3H]TCP binding (r = 0.96) binding. The results are summarized in Table I.

Specificity of the ES-242s in Receptor Binding—ES-242s

Cyclic GMP Accumulation in Rat Cerebellar Slices—The immature cerebella dissected from Sprague-Dawley rats (8 or 9 days old) were cut manually into 0.5-0.8-mm slices in both the sagittal and coronal planes. The tissues were cut again to give pieces that contain approximately 1 mg of protein. The pieces were incubated at 37 °C in a Krebs-Henseleit solution containing 118 mM NaCl, 4.75 mM KH2PO4, 2.54 mM CaCl2, 1.19 mM MgSO4, 1.19 mM KH2PO4, 25 mM NaHCO3, and 10 mM glucose, continuously gassed with 5% CO2, 95% O2. The slices were preincubated for more than 1.5 h. Test compounds were then added and incubated for 3 min (when kainate was added) or 5 min (when L-glutamate or NMDA was added). Then the pieces were removed, put into 500 μl of 50 mM Tris, 4 mM EDTA buffer (pH 7.6), and boiled for 5 min. After tritrogenization with an Eppendorf homogenizer for 5 min, followed by centrifugation at 12,000 × g, the cyclic GMP content of the supernatant fractions were measured by radioimmunoassay. Proteins were analyzed using biocinchoninic acid (41) with bovine serum albumin as a standard.

Neuroprotective Assay—The exposure of cultured hippocampal neurons to drugs was done as described by Favaron et al. (37). Before application of L-glutamate, the culture medium was collected, and the cells were washed with Locke’s solution (154 mM NaCl, 3.6 mM KCl, 3.6 mM NaHCO3, 2.3 mM CaCl2, 1 mM MgCl2, 5.6 mM glucose, and 5 mM HEPES, pH 7.4). After exposure to drugs, the cells were washed again with Locke’s solution and cultured in the conditioned medium for 1 more day at 37 °C. The viability of neurons was estimated by staining with fluorescein diacetate/propidium iodide as described by Favaron et al. (37).
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**Fig. 7.** The effect of ES-242-1 on NMDA- or L-glutamate-induced cyclic GMP accumulation in cerebellar slices. Inhibition of the cyclic GMP response to 100 µM NMDA by 50 µM (NO₂)Arg (A), 1 mg/ml hemoglobin or 20 µM methylene blue (B), or various concentrations of ES-242-1 (C). D, inhibition of cyclic GMP formation induced by 3 mM L-glutamate by various concentrations of ES-242-1. Data represent mean ± S.E. (bars) from four or five slices. Statistically significant differences are indicated: *p < 0.05, **p < 0.01, ***p < 0.001.

**Fig. 8.** The effect of ES-242-1 on kainate- or sodium nitroprusside-induced cyclic GMP accumulation in cerebellar slices. Inhibition of the cyclic GMP response to 100 µM kainate (A), or 10 µM sodium nitroprusside by 10 µM ES-242-1 (B). Data represent mean ± S.E. (bars) from four or five slices. Statistically significant differences are indicated: **p < 0.01, ***p < 0.001.
These compounds were also not effective against the binding of either [3H]kainate or [3H]AMPA, both of which are non-NMDA receptor ligands. Thus, no effect of ES-242s was observed on [3H]TCP binding. Substitution at the 4- and the 4′-positions may cause some conformational change in these molecules, and such a conformational difference may explain the difference in the inhibitory potency of the ES-242s against [3H]TCP binding. Substitution at the 4- and the 4′-positions may cause some conformational change in these molecules, and such a conformational difference may explain the difference in the inhibitory potency of the ES-242s against [3H]TCP binding. Substitution at the 4- and the 4′-positions may cause some conformational change in these molecules, and such a conformational difference may explain the difference in the inhibitory potency of the ES-242s against [3H]TCP binding.

**Effect of ES-242-1 on Ca2+ Entry Induced by Various Glutamate Agonists**—An NMDA-induced elevation of the concentration of intracellular free Ca2+ ([Ca2+]i) was observed in primary cultures of mouse fetal hippocampal neurons (Fig. 6A). Both kainate and quisqualate also elevated [Ca2+]i, under the same conditions (Fig. 6B). The elevation of the [Ca2+]i level in NMDA neurons was blocked by 1 μM MK-801. ES-242-1, at a concentration of 10 μM, depressed NMDA-induced Ca2+ influx, whereas it did not inhibit the [Ca2+]i elevation stimulated by kainate or quisqualate (Fig. 6D).

**Effect of ES-242-1 on Cyclic GMP Production in Rat Cerebellar Slices**—In rat cerebellar slices, cyclic GMP accumulation was stimulated by excitatory amino acids and nitroprusside. When the slices were incubated with 100 μM NMDA, the cyclic GMP level increased 2-5-fold. The elevation of cyclic GMP level elicited by either NMDA or L-glutamate was inhibited by 1 mg/ml hemoglobin, 20 μM methylene blue, and 50 μM L-Nω-nitroarginine (L-Nω-Arg) (Fig. 7, A and B). ES-242-1 inhibited both L-glutamate-induced and NMDA-induced cyclic GMP accumulation at concentrations of 0.01-10 μM in a dose-dependent manner (Fig. 7, C and D). ES-242-1 did not inhibit kainate-induced cyclic GMP accumulation even at a concentration of 10 μM (Fig. 8A). Cyclic GMP level was elevated 20-fold by treatment with 10 μM sodium nitroprusside. ES-242-1, even at concentrations as high as 10 μM, failed to prevent cyclic GMP accumulation induced by sodium nitroprusside (Fig. 8B).

**Protective Effect of ES-242-1 against Glutamate-evoked Neurotoxicity in Primary Cultures of Mouse Hippocampal Granule Cells**—We next examined the effect of ES-242-1 against glutamate-evoked neurotoxicity in *vitro* in primary cultures of fetal mouse hippocampal neurons. The necrosis of a majority of cultured hippocampal granule cells was observed after exposure to 100 μM glutamate. MK-801 at 0.01-1.0 μM prevented glutamate-induced neurotoxicity in a dose-dependent manner. ES-242-1 reduced the cell damage caused by glutamate in the same concentration range as did MK-801 (Fig. 9). Thus, the neuroprotective potency of ES-242-1 in cultured hippocampal neurons was almost equal to that of MK-801.

**DISCUSSION**

The ES-242s, which are the first compounds to be isolated from a microbial source and found to act on the channel domain of the NMDA receptor, have novel and unique chemical structures (Fig. 1). Several chemically different compounds that bind to the channel domain of the NMDA receptor have been reported (42): the arylcyclohexamine "dissociative anesthetics," TCP and ketamine; the benzomorphan "sigma opiates," N-allylnormetazocine (SKF10047); the dibenzocyclohepteneimine, MK-801; and a group of diarylguanidines (16). The structures of ES-242s, a series of bioxanthracenes, are quite different from those of the well-known non-NMDA receptor antagonists. It is of interest that the ES-242s are unlike the other NMDA receptor ligands reported so far in that they do not contain a nitrogen atom. The present results suggest that the nitrogen atom may not always be essential for the binding of the compounds to the NMDA receptor.

The ES-242 compounds possess a 10,10′-bioxanthracene structure having two oxanthracene rings connected with a single C–C bond. Minor structural changes cause the big differences in the inhibitory potency of the ES-242s against [3H]TCP binding. Substitution at the 4- and the 4′-positions may cause some conformational change in these molecules, and such a conformational difference may explain the difference in the inhibitory potency of the ES-242s. The 9,9′-diacetylated ES-242 derivatives, diacetyl-ES-242–1, -2, and -3, have a much lower inhibitory potency against [3H]TCP binding to the NMDA receptor, suggesting that the hydroxyl group on the 9- or the 9′-position might be essential for the inhibitory activities. These results may suggest a strategy for the synthesis of more strongly active compounds acting on the NMDA receptor.

ES-242s inhibited [3H]TCP or [3H]MK-801 binding to rat CSM in a dose-dependent manner. Among them, ES-242-1 was the most potent, inhibiting [3H]MK-801 binding with an IC50 of 66 nM, which is 13-fold greater than that of MK-801 and 15-fold smaller than that of ketamine (data not shown). ES-242-1, -2, and -5 also displaced [3H]CPP or [3H]CGS19755 bound to the NMDA receptor, but at higher concentrations than those required for the inhibition of [3H]TCP or [3H]MK-801 binding. However, even at a higher concentration of 10 μM, the ES-242s were not effective against the binding of non-NMDA receptor ligands, such as [3H]kainate or [3H]AMPA. Although it has been known that some drugs such as phencyclidine and SKF10049 act on σ opioid receptors as well as on the MK-801-binding site of the NMDA receptor (43, 44), ES-242-1 failed to inhibit the [3H]TCP binding to σ opioid receptor. Furthermore, ES-242-1 did not act on adenosine A1; adenosine A2; dopamine D1; histamine H1; histamine H2; serotonin 5-HT1A, 5-HT2A, or 5-HT3 receptors. Taken together, we can conclude that the ES-242s are highly selective ligands for the NMDA receptor.

Of particular interest is the finding that ES-242-1 inhibits not only [3H]TCP or [3H]MK-801 binding but also [3H]CGS19755 binding in a competitive manner. There are two possible mechanisms that might explain how ES-242-1 could
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by glutamate but did not block kainate-elicited neuronal interaction with the NMDA receptor without affecting either gest that ES-242-1 selectively blocks the cyclic GMP production elicited by NMDA or L-glutamate through a specific

Recently, the primary structures of the NMDA receptor have been deduced by cloning and sequencing of the complementarv DNA (46). The ES-242s would be useful tools for investigating the molecular pharmacology of NMDA receptor.

Extracellular Ca\(^{2+}\) enters the cell through the activated NMDA receptor-associated ion channel, whereas kainate and quisquulate cause elevation of the [Ca\(^{++}\)]\(_i\) level indirectly (47). Upon application of ES-242-1 with 100 \(\mu\)M NMDA, [Ca\(^{++}\)]\(_i\) initially rose in a similar degree as in the absence of ES-242-1, however, then progressively came down to the unstimulated basal level (Fig. 6A). This phenomenon was also observed when MK-801 was used (40). One possible explanation for the blockade with a lag by ES-242-1 is that ES-242-1 might be mainly acting on the channel domain, as does MK-801, rather than the ligand-binding site, in the presence of a higher concentration of NMDA such as 100 \(\mu\)M. Since the mode of the blockade of the NMDA-induced increase in [Ca\(^{++}\)]\(_i\), by ES-242-1 is considered to be highly correlated to the binding fashion of ES-242-1, the studies on the precise binding site of ES-242-1 on the NMDA receptor are currently under investigation. In any case, ES-242-1 did not affect the [Ca\(^{++}\)]\(_i\) elevation stimulated by kainate or quisquulate, suggesting that ES-242-1 specifically inhibits the increase in [Ca\(^{++}\)]\(_i\), induced by NMDA.

Nitric oxide (NO) is a remarkable regulatory molecule (48); it acts both as a second messenger and as a neurotransmitter and has been implicated in an extraordinarily diverse range of physiological functions. NO is synthesized from L-arginine in a reaction that is catalyzed by NO synthase stimulated indirectly by the activation of NMDA. The NO produced activates soluble guanylyl cyclase and consequently leads to the formation of cyclic GMP. In the present study, cyclic GMP production was stimulated by excitatory amino acids in rat cerebellar slices. The extent of the stimulation of cyclic GMP production was 10-20 fold less than that reported previously (49). But the elevation of the cyclic GMP level elicited by NMDA was inhibited by hemoglobin (NO trapping agent), (NO\(_2\))Arg (an inhibitor of NO synthase), and methylene blue (an inhibitor of soluble guanylyl cyclase), suggesting that the increase in cyclic GMP production observed under the present experimental conditions occurs through the activation of NO synthase coupled to the NMDA receptor. ES-242-1 inhibited both L-glutamate and NMDA-induced cyclic GMP formation, but was not effective on either kainate or sodium nitroprusside-induced cyclic GMP accumulations. These results suggest that ES-242-1 selectively blocks the cyclic GMP production elicited by NMDA or L-glutamate through a specific interaction with the NMDA receptor without affecting either NO synthase or soluble guanylyl cyclase.

L-Glutamate has been known to exert a toxic effect (4). MK-801 has been shown to prevent glutamate neurotoxicity (5, 50). ES-242-1 selectively reduced the cell damage evoked by glutamate but did not block kainate-elicted neuronal death. The potency of the neuroprotective effect of ES-242-1 on cultured hippocampal neurons was almost equal to that of MK-801, although the inhibitory potencies of ES-242-1 for the binding of [H]TCP or [H]MK-801 are 20-fold lower than those of MK-801.

In conclusion, the ES-242s are a series of NMDA antagonists from the culture broth of Verticillium sp. novel in the sense that: (i) unlike the other NMDA receptor ligands reported so far, ES-242s do not contain nitrogen; (ii) they are effective at antagonizing the binding of both [H]MK-801 and [H]CGP31358, suggesting that they interact with both the neurotransmitter recognition site and the ion channel domain of the NMDA receptor; (iii) ES-242-1 selectively blocked the Ca\(^{2+}\) entry and the cyclic GMP accumulation induced by NMDA; and (iv) importantly, ES-242-1 prevented the neuronal death induced by L-glutamate in cultures of hippocampal neurons. Thus, the ES-242s are a novel type of chemical entity that may provide a useful tool with which to understand the molecular pharmacology of the NMDA receptor and that may possess neuroprotective properties useful in the treatment of diseases involving glutamate toxicity.

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