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EFFECTS OF A NOVEL *N*-METHYL-D-ASPARTATE (NMDA) RECEPTOR ANTAGONIST, 3,3′-DIMETHYL-3,4,3′,4′-TETRAHYDRO-6,8,6′,8′-TETRAMETHOXY-[10,10′-BI-2-OXANTHRACENE]-4,9,9′-(1*H*,1′*H*)-TRIOL 4-ACETATE (ES-242-1), ON NMDA-INDUCED INCREASES OF INTRACELLULAR Ca^{2+} CONCENTRATION IN CULTURED HIPPOCAMPAL NEURONS

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Abstract—The effects of a novel *N*-methyl-D-aspartate (NMDA) receptor antagonist, ES-242-1 (3,3′-dimethyl-3,4,3′,4′-tetrahydro-6,8,6′,8′-tetramethoxy-[10,10′-bi-2-oxanthracene]-4,9,9′-(1*H*,1′*H*)-triol 4-acetate), on NMDA-induced increases of intracellular Ca^{2+} concentration in cultured hippocampal neurons were examined. ES-242-1 selectively blocked the NMDA-induced increase in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$), but not the $[Ca^{2+}]_i$ increase stimulated by quisqualate or kainate. The effect of ES-242-1 appeared in the slow development of a blockade of $[Ca^{2+}]_i$ (half blocking time: 90 sec) when 100 μM NMDA was applied with 10 μM ES-242-1, whereas the initial $[Ca^{2+}]_i$ rise was attenuated by 10 μM ES-242-1 when the latter was applied with a lower concentration of NMDA (10 μM). This is consistent with a previous observation that ES-242-1 binds to both the transmitter recognition site and the channel domain. The blockade by ES-242-1 was reversed by washing. In contrast, the blockade by MK-801 was not relieved easily by washing. These results suggest that ES-242-1 blocks the NMDA-induced $[Ca^{2+}]_i$ increase due to a combination of two well-recognized mechanisms, which are different from that of MK-801, at the NMDA receptor.

Key words: NMDA receptor; antagonist; Ca^{2+} influx; ES-242-1; MK-801; hippocampal neuron

In the mammalian central nervous system, glutamate is a major excitatory neurotransmitter, and produces its effects by acting at various types of receptors [1, 2]. The receptors that directly activate ion channels through agonist stimulation are classified into three major subtypes: those which prefer (1) NMDA†, (2) AMPA or (3) kainic acid as agonists. Activation of these receptors causes an increase in $[Ca^{2+}]_i$ [3–8]. In the CA1 region of the mammalian hippocampus, the $[Ca^{2+}]_i$ rise induced by NMDA receptor activation triggers an activity-dependent change in synaptic efficacy [9–12], known as long-term potentiation (LTP) [13]. LTP is thought to provide the physiological basis for one mechanism of learning and memory in the mammalian brain [14].

On the other hand, it is known that cell injury in the brain associated with anoxia, stroke, hypoglycemia, epilepsy and other neurodegenerative illnesses, such as Huntington's disease, may be partially induced by excessive activation of NMDA receptors [15–17] in a Ca^{2+}-dependent manner [17–19]. Therefore, it has been suggested that NMDA receptor antagonists, such as D-AP5 (D-2-amino-5-phosphonovalerate) [20], CGS 19755 (*cis*-4-phosphonomethyl-2-piperidine carboxylic acid) [21], CPP [3-3(2-carboxypiperazine-4-ly)propyl-1-phosphate] [22], TCP [1-(1-thienly-cyclohexyl)]piperidine [23], and MK-801 {(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate} [24], could protect neurons from brain damage [25–30].

3,3′ - Dimethyl - 3,4,3′,4′ - tetrahydro - 6,8,6′,8′ - tetramethoxy - [10,10′ - bi - 2 - oxanthracene] - 4,9,9′-(1*H*,1′*H*)-triol 4-acetate (ES-242-1), a novel bioxanthracene derivative isolated from the culture broth of *Verticillium* sp. [31], has been found to be a potent and specific NMDA receptor antagonist [32]. The structure of ES-242-1 is quite different from those of the well-known NMDA receptor antagonists. It is of interest that ES-242-1 is unlike the other NMDA receptor ligands reported thus far in that it does not contain a nitrogen atom. Another characteristic of ES-242-1 is that it inhibits the binding of both [^{3}H]TCP and [^{3}H]CGS 19755 in a competitive manner, suggesting that ES-242-1 interacts with both the transmitter recognition site and the channel domain. There are no reports of other compounds

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† Abbreviations: NMDA, *N*-methyl-D-aspartate; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; TCP, [1-(1-thienly-cyclohexyl)] piperidine; BSS, basal salt solution.

that act in this manner. Because of our previous observation [32] that the antagonistic properties of ES-242-1 appear to be due to a novel mechanism of action at the NMDA receptor, we were interested in exploring the effects of ES-242-1 on the NMDA-induced increase in $[Ca^{2+}]_i$ in cultured hippocampal neurons. In addition, we compared the inhibitory profile of ES-242-1 with that of MK-801, the most potent and the best characterized non-competitive NMDA receptor antagonist reported to date [24].

MATERIALS AND METHODS

Materials. ES-242-1 was purified from the culture broth of *Verticillium* sp. as reported [31]. MK-801 was synthesized chemically by Dr. H. Obase and Mr. H. Kato in the Pharmaceutical Research Laboratories of our company. NMDA, quisqualic acid, nicardipine and tetrodotoxin were obtained from the Sigma Chemical Co. All other chemicals were of analytical grade.

Primary culture of mouse embryonic hippocampal neurons. Pregnant ICR mice (Charles River Japan) were killed by exposure to ethyl ether on day 16–19 of gestation. The embryonic offspring were removed, and hippocampal tissues were dissected from the brains of fetuses and placed into Ca^{2+}/Mg^{2+}-free Hanks' balanced salt solution (GIBCO). These tissues were treated for 13 min at 37° with 0.25% trypsin (Sigma) and 0.01% DNase I (Sigma) followed by an additional treatment with 0.01% DNase I for 2 min at 37°. Then, the dissociated cells were washed with culture medium containing 10% horse serum and were dispersed by repeated pipetting. The cells were collected by centrifugation at 200 *g* for 4 min, and resuspended in serum-free Dulbecco's modified Eagle's medium [33] (Nissui; containing no L-glutamate) supplemented with 1 mg/mL bovine serum albumin, 10 μg/mL insulin, 0.1 mg/mL transferrin, 0.1 nM L-thyroxine, 30 nM sodium selenide (all from Sigma), 1 μg/mL aprotinin (Funakoshi) and 0.1 mg/mL streptomycin–100 U/mL penicillin (GIBCO). The suspended cells were plated on a poly-L-lysine (Sigma) coated glass coverslip (Matsunami) with a silicon rubber wall (Heraeus, Flexiperm) at a density of $1.8–2.0 \times 10^5$ cells/cm^2. The culture was maintained for 11–13 days with the culture medium described above in a humidified atmosphere of 5% CO_2 in air at 37°.

Measurement of $[Ca^{2+}]_i$. Culture medium was removed, and the cells on the coverslip were washed at least three times with BSS (composition in mM: NaCl, 130; KCl, 5.4; $CaCl_2$, 1.8; D-glucose, 5.5; HEPES-NaOH, 20; pH was adjusted to 7.3). Fura-2/AM (5 μM) (Dojin) in BSS was then incubated with the cells for 60 min at 37°.

The coverslip with the cultured neurons that had been loaded with fura-2 was placed on the stage of an inverted microscope (Nikon) equipped with a UV-fluor objective (Nikon; ×10), and the cells were illuminated by the beam from a xenon lamp at an excitation wavelength of 340 or 380 nm (bandwidth; 10 nm). One set of fluorescence images was collected through an emission filter (490-nm long pass filter) with a silicon-intensified-target video camera (Hamamatsu Photonics) every 10 or 15 sec. One-quarter of the area of the analogue-output image from the camera was mapped onto 128 × 120 pixels (total magnification: ×200), and the fluorescence intensity of each pixel was translated into an 8-bit digital datum. The data obtained from each pair of fluorescence images were stored temporarily as frame memories of a video-analyzing system (Hamamatsu Photonics, ARGUS), and were then filed on the hard disk of the host computer (Toshiba, J-3300). After subtracting the background from the pair of digital images, fluorescence-ratio images (F340/F380) were calculated using an Argus200 (Hamamatsu Photonics) software system.

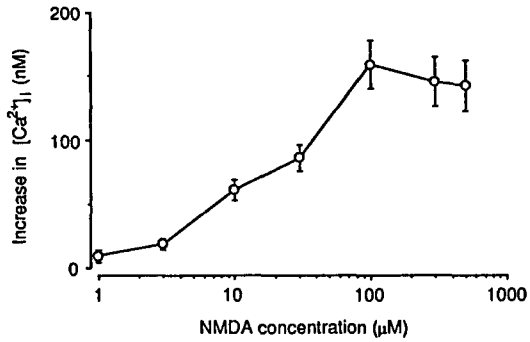

Fig. 1. Effect of increasing concentration of NMDA on $[Ca^{2+}]_i$ in cultured mouse hippocampal neurons. The concentration–response curve for NMDA was plotted as the $[Ca^{2+}]_i$ increase for 30 sec from resting $[Ca^{2+}]_i$ against the log of the NMDA concentration. Values are means ± SEM for 3 cells.

Neurons were discriminated from glia and other cells by their brighter fluorescence and the presence of smooth, long, straight processes as described by Kudo and Ogura [34]. The temporal data from a single cell soma derived from ratio images were analyzed with the host computer. The concentration of Ca^{2+} was estimated by comparison with the fluorescence intensity ratios of Ca^{2+}-EGTA mixtures in (3-(*N*-morpholino) propanesulfonic acid (MOPS) buffer added to 10 μM fura-2 and excited at the two wavelengths, as described by Grynkiewicz *et al.* [35]. Experiments were performed at 34° except where otherwise stated.

Drug application. Neurons were superfused continuously with BSS through two capillary tubes placed just above the coverslips at a flow rate of approximately 2 mL/min. The volume of the medium was maintained at about 0.5 mL. Drugs were added to the superfusing medium. Tetrodotoxin (0.5 μM) was always included in the BSS in order to block synaptic activity [36]. In all experiments Mg^{2+}, Zn^{2+}, glycine and polyamines were not added to the BSS. Data are expressed as means ± SEM.

RESULTS

NMDA-induced $[Ca^{2+}]_i$ *increase.* In cultured mouse hippocampal neurons loaded with fura-2, the mean value of resting $[Ca^{2+}]_i$ was 41 ± 1 nM (N =

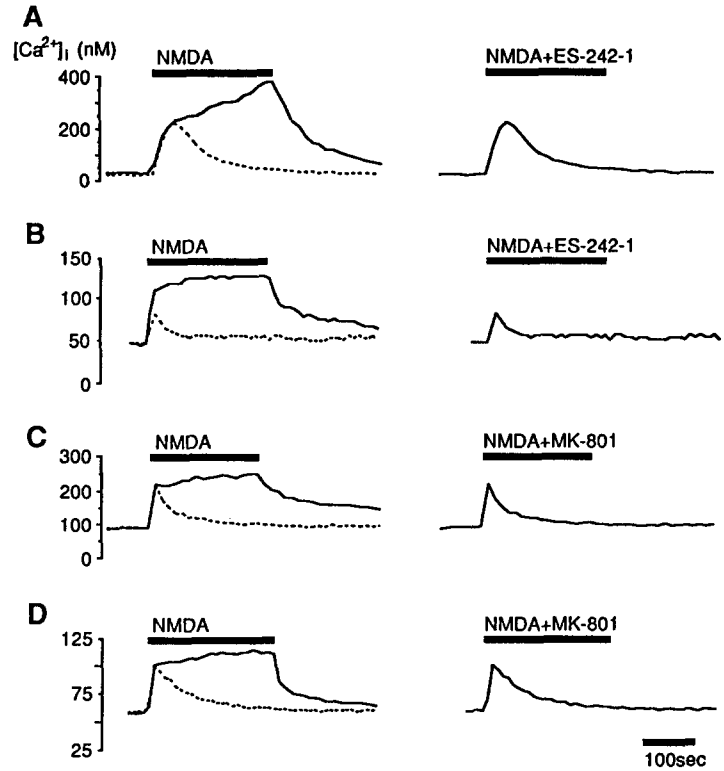

Fig. 2. Blockade by ES-242-1 of NMDA-induced $[Ca^{2+}]_i$ elevation. The effects of ES-242-1 (10 μM) on the Ca^{2+} increase induced by 100 μM NMDA (A) or 10 μM NMDA (B), and the effect of 1 μM MK-801 on the Ca^{2+} increase induced by 100 μM NMDA (C) or 10 μM NMDA (D) were examined. The $[Ca^{2+}]_i$ elevation was measured in the absence (left column) or presence (right column) of ES-242-1 or MK-801. Superimposed broken lines are the same data as the right column. The black horizontal bars indicate the period of the application of the stimulant alone or together with ES-242-1 or MK-801. The camera output was transferred to a host computer (Toshiba, J-3300) at sampling intervals of 15 sec. Data represent the mean values of 6 (A), 12 (B), 17 (C) and 14 (D) cells.

260). This value is similar to those reported in the literature [4–6, 33, 34]. NMDA induced an increase in $[Ca^{2+}]_i$ in a concentration-dependent manner in a range from 1 to 100 μM within 30 sec (Fig. 1); the maximal response was obtained at 100 μM NMDA. In the following experiments, then, NMDA was applied at a concentration of 100 μM.

Blockade by ES-242-1 of NMDA-induced $[Ca^{2+}]_i$ *increase.* Figure 2 shows the blockade by ES-242-1 of NMDA-induced $[Ca^{2+}]_i$ increase. Application of 100 μM NMDA for 4 min caused $[Ca^{2+}]_i$ to increase to 346 ± 42 nM (N = 6, control value) (Fig. 2A, left trace). By contrast, upon application of 10 μM ES-242-1 in combination with 100 μM NMDA, $[Ca^{2+}]_i$ initially increased to a similar degree as it did in the absence of ES-242-1 (93 ± 17% of control); however, then $[Ca^{2+}]_i$ progressively came down to the unstimulated basal level (Fig. 2A, right trace). The same phenomenon was observed when 1 μM MK-801 was applied in combination with 100 μM NMDA (Fig. 2C); this is consistent with previous findings in the literature [33]. When the concentration of NMDA applied was lowered to 10 μM, the initial $[Ca^{2+}]_i$ elevation was clearly inhibited by 10 μM ES-242-1 (Fig. 2B; 56 ± 2% of control), but not by MK-801 (Fig. 2D). These data are consistent with the interpretation that ES-242-1 interacts with the agonist binding site as well as the channel domain of NMDA receptors (see Discussion). On the other hand, when ES-242-1 was applied in combination with quisqualic acid or kainic acid, ES-242-1 did not change significantly the size or the time course of the responses (data not shown) as reported previously [32].

Concentration-dependent blockade by ES-242-1. The NMDA-induced $[Ca^{2+}]_i$ increase was measured in the presence of various concentrations of ES-242-1 (Fig. 3A). Neurons were superfused with BSS containing 10 μM nicardipine in order to avoid effects of voltage-dependent Ca^{2+} channels. NMDA at 100 μM and different concentrations of ES-242-1 were applied simultaneously for 7 min. The response to 100 μM NMDA alone was measured as a control. After an equilibrium blocking period by ES-242-1 (6.75 min after the drug application), the degree of blockade of the response to NMDA by ES-242-1 was calculated (Fig. 3B). ES-242-1 inhibited the NMDA-induced $[Ca^{2+}]_i$ increase by 13 ± 9 (N = 13), 31 ± 9 (N = 18), 70 ± 5 (N = 19), 90 ± 2 (N = 20), 98 ± 1 (N = 19), and 96 ± 1% (N = 21) at concentrations of 0.03, 0.1, 0.3, 1, 3, and 10 μM, respectively; the IC_{50} value was calculated to be 0.16 μM.

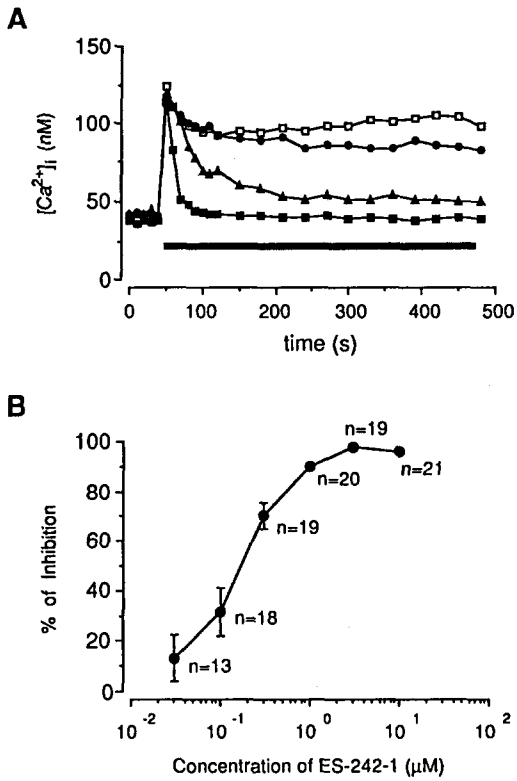

Fig. 3. Concentration–response curve for the inhibition of NMDA-induced $[Ca^{2+}]_i$ elevation by various concentrations of ES-242-1. (A) Neurons were superfused with BSS containing 10 μM nicardipine in order to avoid the effects of voltage-dependent Ca^{2+} channels. A 100 μM concentration of NMDA and 0 (□), 0.1 (●), 1.0 (▲) or 10 μM (■) ES-242-1 were applied simultaneously for 7 min. The black horizontal bar indicates the period of the application of NMDA alone or together with ES-242-1. (B) The percent inhibition of the blockade of the response to NMDA in the presence of various concentrations of ES-242-1 was calculated after an equilibrium blockade by ES-242-1 was achieved (6.75 min after the drug application). The response to 100 μM NMDA alone was measured as a control. Data are the means ± SEM of the response of 13–21 cells.

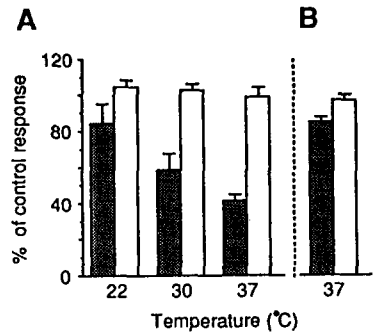

Fig. 4. Effect of temperature on the blockade by ES-242-1 of NMDA-induced $[Ca^{2+}]_i$ elevation. After the control responses to 100 μM NMDA or 5 μM quisqualic acid were obtained, 10 μM ES-242-1 was applied alone for 5 min (A) or no drugs were applied (B). The responses to subsequent application of NMDA and quisqualic acid were normalized as percent of the control response. Absolute values of control response to NMDA and quisqualic acid are listed in Table 1. Shaded and open bars indicate NMDA and quisqualic acid responses, respectively. Each bar represents the mean ± SEM of the response of 8 and 24 cells for A and B, respectively.

Table 1. NMDA or quisqualic acid (QA) responses at various temperatures

	$\Delta[Ca^{2+}]_i$ (nM)	
Temperature	NMDA, 100 μM	QA, 10 μM
22°	89.6 ± 21.1	186.4 ± 31.1
30°	80.2 ± 15.8	192.4 ± 31.7
37°	96.8 ± 18.0	192.6 ± 41.3

The increase in intracellular Ca^{2+} concentration ($\Delta[Ca^{2+}]_i$) caused by NMDA or quisqualate was measured as described in the legend to Fig. 3, but without ES-242-1. Data are means ± SEM of 8 cells. The data were evaluated with an ANOVA test.

Effect of temperature on the blockade by ES-242-1. Since the blockade of the response to NMDA by MK-801, the most potent non-competitive NMDA receptor antagonist reported thus far, is temperature dependent [33], it was of interest to know whether the inhibition by ES-242-1 also has a temperature-dependent component. Accordingly, we measured the blockade by ES-242-1 at different temperatures in the same experimental design as reported using MK-801 [33]. After the control responses to NMDA (100 μM, 30 sec) or quisqualic acid (5 μM, 30 sec) were obtained, 10 μM ES-242-1 was applied alone for 5 min. After the neurons were washed with normal BSS for 10 min, the subsequent response to NMDA was measured; the response was reduced to 84 ± 11, 59 ± 9 and 41 ± 4% of the control at 22°, 30°, and 37°, respectively (Fig. 4A; shaded bars, N = 8 for each temperature). On the other hand, the response to quisqualic acid was not altered by ES-242-1 at any temperature under these conditions; the percent value of the control response to quisqualic acid was 105 ± 4, 102 ± 4, and 99 ± 5% at 22°, 30°, and 37°, respectively (Fig. 4A; open bars, N = 8 for each temperature). To avoid the possibility that the desensitization of the receptor channel occurred at higher temperatures, a drug-free test was conducted. The responses to NMDA and quisqualic acid after washing for 10 min without application of ES-242-1 were reduced to only 85 ± 3 and 98 ± 4% of the control, respectively, even at 37° (Fig. 4B, N = 28). Furthermore, no alteration of the magnitude of the $[Ca^{2+}]_i$ rise following NMDA or quisqualate application was observed at any of the three different temperatures (Table 1). The temperature-sensitive nature of the blockade by ES-242-1 is thus the same as that reported for MK-801 [33].

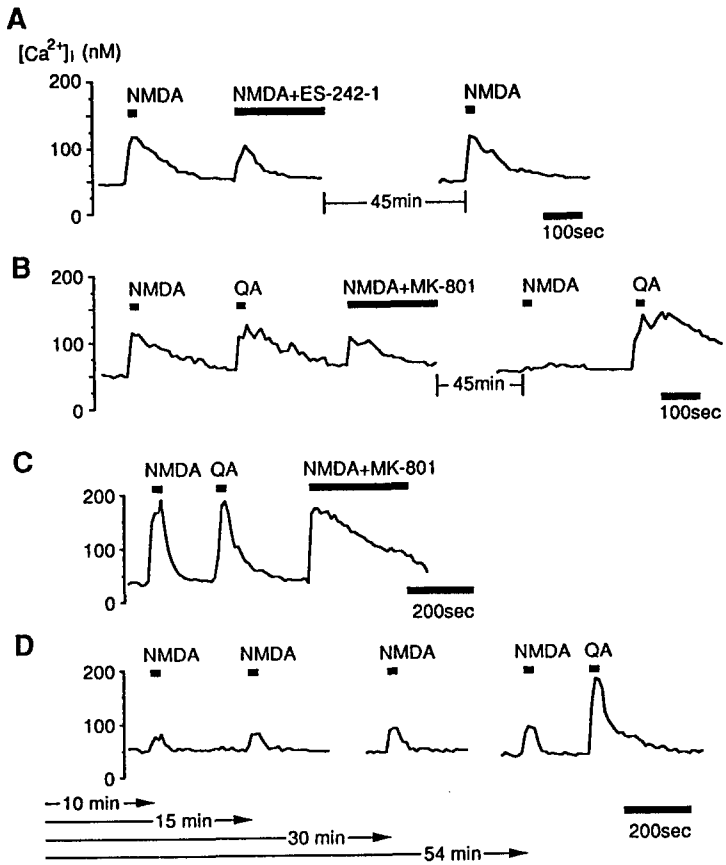

Fig. 5. Recovery from the blockade by ES-242-1 or MK-801 of NMDA-induced $[Ca^{2+}]_i$ elevation. Recovery of NMDA-induced $[Ca^{2+}]_i$ elevation from the blockade by ES-242-1 was compared with that by MK-801. (A) After the initial response to 100 μM NMDA was obtained, 10 μM ES-242-1 was applied in combination with 100 μM NMDA. The subsequent response to 100 μM NMDA was traced after washing with superfusing medium for 45 min. (B) After the initial responses to 100 μM NMDA or 5 μM quisqualic acid (QA) were obtained, 1 μM MK-801 was applied in combination with 100 μM NMDA. The subsequent responses to 100 μM NMDA or 5 μM quisqualic acid were traced after 45 min of washing. (C) All procedures were the same as those described above except that the concentration of MK-801 was reduced from 1.0 to 0.1 μM. (D) NMDA at 100 μM was applied repeatedly at the indicated time (arrows) during the washing period after the application of 0.1 μM MK-801 in combination with 100 μM NMDA. The black horizontal bars indicate the period of the application of the stimulant alone or together with inhibitors. Data represent the mean values of 6 (A and B) and 10 (C and D) cells.

Recovery from blockade. As described above, we found that the blockade by ES-242-1 was not relieved by 10 min of washing at physiological temperatures. We next asked if the blockade by ES-242-1 continues for more than 10 min. Figure 5 shows that ES-242-1 almost completely blocked (97 ± 5% inhibition) the sustained response to NMDA excepting the initial $[Ca^{2+}]_i$ increase, as did MK-801 (94 ± 3% inhibition), and that the initial increase in $[Ca^{2+}]_i$ by ES-242-1 at 10 μM was observed again after washing the cells with BSS for 45 min. The subsequent response to NMDA was 94 ± 1% (N = 6) of the initial response (Fig. 5A). By contrast, the blockade by MK-801 was not reversed by 45 min of washing; the subsequent response of the initial $[Ca^{2+}]_i$ increase to NMDA was only 16 ± 3% (N = 6) of the initial response (Fig. 5B). The subsequent response to quisqualic acid was found to be almost the same as the initial response after the MK-801 treatment, suggesting that the neurons were not damaged. The blockade by MK-801 was long-lasting, even when the concentration of MK-801 was lowered from 1.0 to 0.1 μM. The blockade by MK-801 was not recovered by washing in the absence of NMDA (data not shown) and was partially and slowly reversed by repeated applications of NMDA (Fig. 5, C and D). This characteristic of MK-801 is consistent with a previous observation by Yuzaki *et al.* [33]. The results presented here clearly show that the blockade by ES-242-1 recovers much faster than that by MK-801.

DISCUSSION

We have investigated the action of ES-242-1 on agonist-induced increases in $[Ca^{2+}]_i$ in cultured

mouse hippocampal neurons. Microfluorometry with the Ca^{2+} indicator dye fura-2 was used to measure the $[Ca^{2+}]_i$. ES-242-1 selectively blocked NMDA-induced $[Ca^{2+}]_i$ increase, but did not block the $[Ca^{2+}]_i$ increase produced by quisqualic acid or kainic acid. This characteristic of ES-242-1 as a specific NMDA receptor antagonist is consistent with previous observations, indicating that ES-242-1 has no effect on the binding of specific ligands for non-NMDA receptors, such as quisqualic acid and kainic acid; in addition, 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 synthetase or soluble guanylyl cyclase [32]. Also, the data presented here probably can explain the attenuation of glutamate-induced neuronal death by ES-242-1 in cultures of hippocampal neurons [37]. Indeed, ES-242-1 inhibited the NMDA-induced $[Ca^{2+}]_i$ increase in a concentration-dependent manner in a dissociated neuronal cell culture. The IC_{50} was calculated to be 0.16 μM (Fig. 3B). This IC_{50} value was similar to that (0.116 μM) determined in binding experiments with [^{3}H]TCP, a ligand for the channel domain of NMDA receptors [32]. Since high concentrations of NMDA (100 μM) were used, this IC_{50} value presumably reflects mainly the inhibitory effect of ES-242-1 on the channel domain. Of course, further experiments designed to measure activity at each site independently and exactly are needed.

As described in a previous paper [32], of particular interest is the finding that ES-242-1 inhibits the binding of not only [^{3}H]TCP and [^{3}H]MK-801 but also [^{3}H]CGS 19755 in a competitive manner. However, the binding affinity of ES-242-1 for the agonist binding site is 10-fold less than that for the ion channel domain. There has been no previous report of a compound with properties similar to those of ES-242-1. Although a mechanism that might explain how ES-242-1 could competitively inhibit the binding of these two ligands is not clear, at least we can say that ES-242-1 interacts with both the transmitter recognition site and the channel domain. This suggestion is supported by the present study, since the mode of the blockade of the NMDA-induced $[Ca^{2+}]_i$ increase by ES-242-1 is considered to be highly correlated to the binding properties of ES-242-1. Upon application of ES-242-1 with 100 μM NMDA, $[Ca^{2+}]_i$ initially increased to a degree similar to that seen in the absence of ES-242-1, but then decreased to the unstimulated basal level (Fig. 2A). This phenomenon was also observed when MK-801 (Fig. 2C), which is known to interact with the ion channel domain [33], was used. It has been reported that MK-801 binding to the NMDA receptor was enhanced by the simultaneous presence of excitatory amino acid agonists [38]. A possible explanation for the slow development of the blockade by ES-242-1 is that ES-242-1 in the presence of a higher concentration of NMDA, such as 100 μM, may be acting mainly on the channel domain, as does MK-801, rather than on the ligand binding site. If so, since we claim that ES-242-1 also binds to the agonist binding site, there should not be a lag when the applied concentration of NMDA was lowered or the concentration of ES-242-1 was increased. Because of a low solubility of ES-242-1, we adopted the former conditions. Indeed, the initial increase in $[Ca^{2+}]_i$ was clearly diminished by ES-242-1 when the applied concentration of NMDA was lowered from 100 to 10 μM (Fig. 2B), but was not diminished by MK-801 (Fig. 2D).

That the blockade is temperature dependent is a property that is common to ES-242-1 and MK-801 in the blockade of an increase in $[Ca^{2+}]_i$ in response to NMDA [33]. On the other hand, the greatest difference in the inhibitory profile between the two compounds is the reversibility of the blockade. The blockade by MK-801 at 1.0 μM was long-lasting and was not reversed completely by the 45-min washing. When MK-801 was applied at 0.1 μM followed by repeated applications of NMDA, a slow recovery was observed, as reported previously [33, 39]. By contrast, the blockade by ES-242-1 at 10 μM was easily reversed by washing in the absence of NMDA. Although the data presented here support the fact that the mode of blockade by ES-242-1 is different from that observed with MK-801, the mechanistic differences are still speculative and require study. For example, the more rapid reversibility by ES-242-1 could simply be due to different lipid solubilities between ES-242-1 and MK-801. In any case, the determination of the precise binding site of ES-242-1 on the NMDA receptor is a necessary part of any effort to clarify the complicated mechanism of action of ES-242-1. To achieve this purpose and to confirm our findings presented here, a more direct and powerful approach, such as patch clamping, is required. Further, the recent progress in determining the primary structures of the NMDA receptor by cloning and sequencing of the complementary DNA [40] will also be helpful for this purpose.

In conclusion, ES-242-1 specifically and reversibly blocked the increase in $[Ca^{2+}]_i$ stimulated by NMDA due to a combination of two well-recognized mechanisms, which are different from that of MK-801, at the NMDA receptor. ES-242-1 is a unique type of NMDA receptor antagonist that may provide a useful tool with which to understand the biochemical and pharmacological properties of the NMDA receptor.

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