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Eosine-Induced Blockade of *N*-Methyl-D-aspartate Channels in Acutely Isolated Rat Hippocampal Neurons

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MOLECULAR PHARMACOLOGY

ABSTRACT

Acutely isolated rat hippocampal neurons were voltage-clamped in the whole-cell configuration. The currents through *N*-methyl-D-aspartate (NMDA) channels were elicited by fast application of aspartate in a Mg^{2+}-free 3 μM glycine-containing solution. Eosine, known as a potent reversible inhibitor of the plasma membrane Ca^{2+} pump, proved to be able to induce a blockade of NMDA channels. The eosine-induced inhibition of NMDA-mediated currents enhanced with eosine concentration ($IC_{50} = 248$ μM) but did not depend on the membrane potential, agonist (aspartate) or coagonist (glycine) concentrations, pH, or the presence of spermine, ethanol, and the disulfide-reducing agents dithiothreitol and glutathione. Zn^{2+} inhibited NMDA channels with equal efficiency both in the presence and absence of eosine. These results suggest that eosine interacts with a new, previously unknown NMDA receptor regulatory site.

N-methyl-D-aspartate (NMDA) receptor channels play an important role in the generation of rhythmic motor activity, regulation of neuronal development, synaptic plasticity, as well as in various processes associated with learning, memory, and cell death (see reviews by McBain and Mayer, 1994; Dingledine et al., 1999). They are also thought to be implicated in a variety of neurologic disorders such as epilepsy, ischemia, Parkinson's and Alzheimer's diseases, amyotropic lateral sclerosis, and Huntington's chorea. Such an important physiological role of NMDA receptors is accompanied by their complex regulation. Being more selective for Ca^{2+} than for monovalent cations, NMDA channels are blocked by extracellular Mg^{2+} in a strongly voltage-dependent manner. A number of regulators including divalent and heavy metals, protons, amino acids (glycine (GLY), alanine), fatty acids, polyamines, reducing/oxidizing agents, and alcohols were reported to modulate NMDA receptor activity.

The present study demonstrates that the list of NMDA channel blockers should be supplemented with one more compound, eosine (tetrabromofluoresceine, EOS; Fig. 1), known in cell biology as a potent ($IC_{50} \approx 1$ μM) inhibitor of the plasma membrane Ca^{2+} pump (Gatto et al., 1995). The action of EOS on NMDA channels proved to be mediated by a new, previously undescribed, inhibitory site. A discovery of this blocking site may provide a new target for medical drugs that could be synthesized for treatment of neurological disorders.

Materials and Methods

Pyramidal neurons were acutely isolated from the CA-1 region of rat hippocampus using "vibrodissociation techniques" (Vorobjev, 1991). The experiments were begun not earlier than after 3-h incubation of the hippocampal slices in a solution containing: 124 mM NaCl; 3 mM KCl; 1.4 mM $CaCl_2$; 2 mM $MgCl_2$; 10 mM glucose; 26 mM $NaHCO_3$. The solution was bubbled with carbogen at 32°C. During the whole period of isolation and current recording, nerve cells were washed with a Mg^{2+}-free solution: 140 mM NaCl; 5 mM KCl; 2 mM $CaCl_2$; 15 mM glucose; 10 mM HEPES; pH 7.3. Three micromolars of GLY was always added to the solution except as noted (see Fig. 4*C*). Fast replacement of superfusion solutions was achieved by using the concentration-jump technique (Benveniste et al., 1990; Vorobjev, 1991). The currents were recorded at 18°C in the whole-cell configuration using micropipettes made from pyrex tubes and filled with an "intracellular" solution: 140 mM CsF; 4 mM NaCl; 10 mM HEPES; pH 7.2. The electrical resistance of the filled micropipettes was 3 to 7 mΩ. Analogous current signals were digitized at 1 kHz frequency.

Statistical analysis was performed using the scientific and technical graphics computer program Microcal Origin (Version 4.1 for Windows, Microcal Software, Inc., Northampton, MA). All the data presented are mean ± S.E.; comparisons were made using the paired Student's t test.

GLY was obtained from Serva (Heidelberg, Germany). Aspartate (ASP), EDTA, EOS, spermine, and dithiothreitol (DTT) were obtained from Sigma (St. Louis, MO).

Results

Ionic currents through NMDA channels were elicited by fast application of 100 μM ASP in a Mg^{2+}-free, 3 μM GLY-

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ABBREVIATIONS: NMDA, *N*-methyl-D-aspartate; EOS, eosine; ASP, aspartate; GLY, glycine; DTT, dithiothreitol.

containing solution. At the holding potential, $E_h = -100$ mV, ASP induced an inward current that, after an initial fast rise ($\tau < 30$ ms) up to the peak value, I_0, decreased gradually ($\tau = 250$–750 ms) down to a certain plateau level, I_C (Fig. 2A, first trace). Such a current decay under continued action of the agonist is considered to be due to desensitization of the NMDA receptor channel complex. The fraction of desensitized channels, $d = 1 - I_C/I_0$, varied between the cells over a wide range of 0.08 to 0.76.

When coapplied with ASP, EOS inhibited NMDA-mediated currents. Figure 2A shows a representative example of currents induced by 3-s ASP (100 μM) coapplication with EOS at different concentrations. During each coapplication, the current reached its stationary level, I_B, which decreased with EOS concentration. If the EOS concentration was smaller than 1 mM, the ASP-induced current was restored completely after EOS removal (cf. the first and sixth traces in Fig. 2A); at higher EOS concentrations the current inhibition became irreversible (cf. first and last traces in Fig. 2A; $I_C{}^*/I_C = 0.35$). The concentration dependence of the degree of stationary current inhibition (I_B/I_C) is shown in Fig. 2B. Fitting of the dose-effect relation by a logistic equation yielded a half-blocking concentration, $IC_{50} = 248 \pm 19$ μM, and a Hill coefficient, $n_H = 1.31 \pm 0.10$ ($n = 10$).

The kinetics of EOS-induced inhibition were studied by applying this compound in the continuous presence of ASP (100 μM). EOS used at different concentrations (62.5–1000 μM) was applied for 2 s after the ASP-induced current reached its stationary level, I_C (Fig. 2C). The onset kinetics were well fitted with double-exponential functions (solid lines). The fast and slow time constants did not depend on EOS concentration, being, on the average, $\tau = 75 \pm 8$ and $\tau = 919 \pm 41$ ms, respectively ($n = 5$). Thus, EOS inhibited ASP-induced currents much faster than it can permeate the cell membrane. According to Gatto et al. (1995), this permeation has a minute-order kinetics. For this reason, it is doubtful that the EOS block can be explained by a potent effect on an intracellular site of the NMDA receptor. Additionally, we also carried out experiments ($n = 3$) including 10 μM EOS in the pipette solution. However, this inclusion did not abolish NMDA receptor currents. Moreover, ASP-induced currents of typical amplitude (1–2 nA) were recorded during time periods typical for other experiments with EOS (20–30 min). Therefore, the existence of a high-affinity intracellular EOS blocking site is highly unlikely.

The EOS-induced inhibition of NMDA channel-mediated currents was practically independent on the holding potential. Figure 3A shows a representative example of currents induced by ASP alone (left traces) and by its coapplication with 100 μM EOS (right traces) at different E_h. Both I_B and I_C values linearly depended on E_h (Fig. 3B). Therefore, the

degree of the stationary current inhibition (I_B/I_C) was approximately the same (0.61) at different E_h. We carried out the experiments analogous to that illustrated in Fig. 3 at different EOS concentrations. The result was the same: the
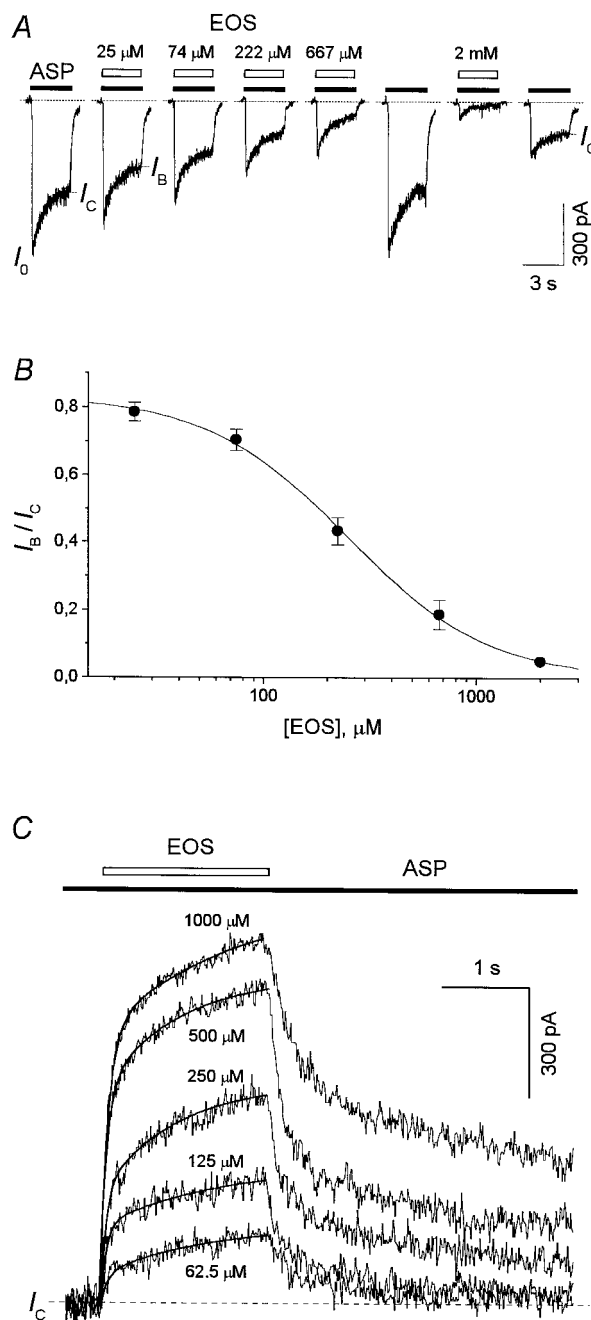

Fig. 2. The concentration dependence and kinetics of the EOS-induced blockade of NMDA channels. A, NMDA responses in the absence (control) and presence of EOS. EOS used at different concentrations (25, 74, 222, 667, and 1000 μM) was coapplied for 3 s with ASP (100 μM). The intervals between the coapplications were 20 to 30 s. B, plateau current magnitudes (I_B) divided by the control plateau value (I_C) were plotted against EOS concentration. The solid line shows the fitting of the experimental data with the logistic equation. The fit parameters are: $IC_{50} = 248 \pm 19$ μM, $n_H = 1.31 \pm 0.10$ ($n = 10$). C, kinetics of EOS-induced inhibition. ASP (100 μM) was applied continuously. EOS (62.5–1000 μM) was coadministered with ASP for 2 s. The onset of EOS-induced inhibition was fitted with double-exponential functions (solid lines) with fast ($\tau = 75 \pm 8$ ms, $n = 5$) and slow ($\tau = 919 \pm 41$ ms, $n = 5$) time constants that were practically independent of EOS concentration.
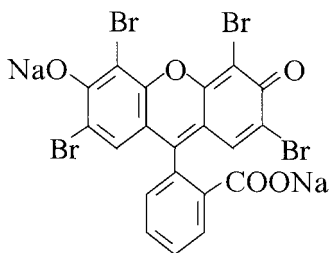

Fig. 1. Chemical structure of EOS (2′,4′,5′,7′-tetrabromofluorescein).

EOS-induced inhibition of currents through NMDA channels was practically voltage independent. All the experiments described below were performed at a holding potential of −100 mV.

The following experiments were carried out to clarify whether EOS acts as a competitive or noncompetitive NMDA receptor channel blocker.

At first, we examined the possibility of EOS competition with ASP for the agonist binding sites on the NMDA receptor. ASP used at different concentrations (6.25, 12.5, 25, 50, and 100 μM) was applied for 3 s without or with 200 μM EOS (Fig. 4A). The mean value of I_B/I_C did not depend on ASP concentration (Fig. 4B), being, on average, 0.51 ± 0.01 ($n = 4$).

Next, we made an attempt to check whether EOS competes with GLY for coagonist binding sites. EOS (200 μM)-induced stationary current inhibition did not vary with GLY concentration either. The mean I_B/I_C value did not depend on GLY concentration (Fig. 4C), being, on average, 0.55 ± 0.01 ($n = 12$).

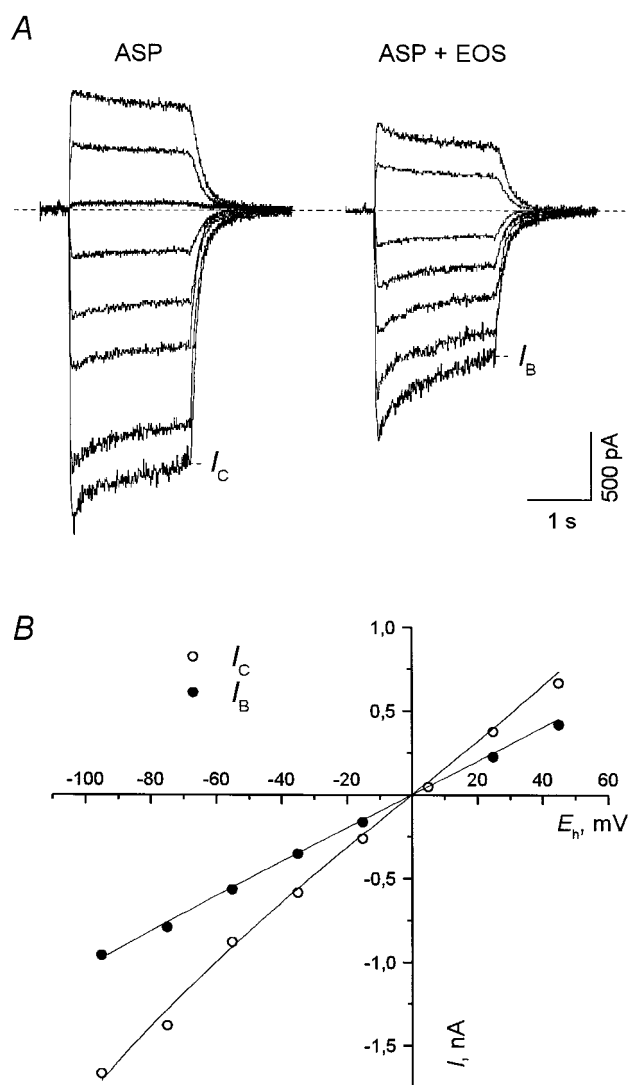The independence of the EOS-induced blockade on ASP and GLY concentrations leaves no doubt that EOS does not compete with either the agonist or the coagonist for common binding sites on the NMDA receptor channel.

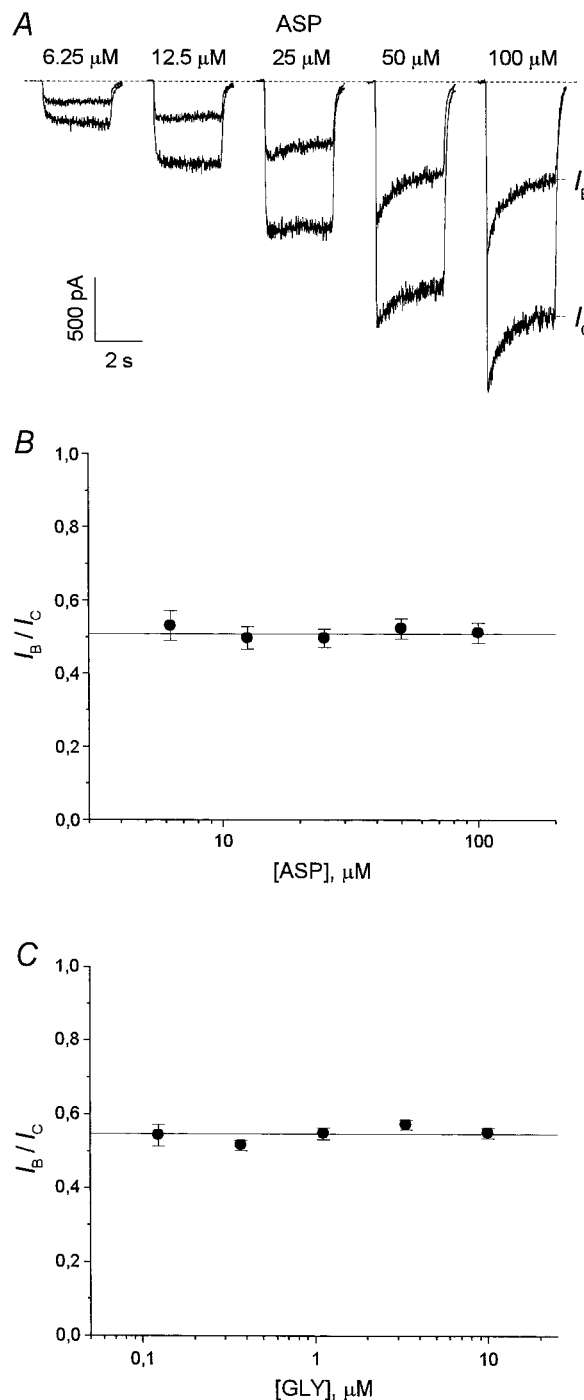The NMDA receptor channel is known to have several

Fig. 4. Agonist (ASP) and coagonist (GLY) independence of the EOS-induced blockade of NMDA channels. A, NMDA responses in the absence and presence of EOS (200 μM) at different concentrations of ASP (6.25, 12.5, 25, 50, and 100 μM). B, mean values of the I_B/I_C ratio were plotted against the ASP concentration. The degree of the stationary EOS-induced current inhibition did not depend on ASP concentration ($n = 4$). C, independence of EOS-induced stationary current inhibition on GLY concentration ($n = 12$). By analogy with A, the NMDA responses were recorded in the absence and presence of EOS (200 μM) at different GLY concentrations (0.12, 0.37, 1.1, 3.3, and 10 μM).

Fig. 3. Voltage independence of the NMDA channels blockade by EOS. A, NMDA responses to 2-s 100 μM ASP application in the absence (left) and presence (right) of EOS (100 μM) at E_h from −95 to 45 mV with a step of 20 mV. B, stationary values of control, I_C, and blocked, I_B, currents were plotted against the holding potential. The ratio, I_B/I_C, was practically independent on E_h.

regulatory sites including redox, proton, Zn^{2+}, and polyamine sites.

To find out whether EOS interacts with the redox modulatory site on the NMDA channel, we tested its effect on 3 mM DTT-treated cells. In our experiments, the kinetics of the 3 mM DTT-induced potentiation was fast. The onset as well as the offset kinetics were well fitted with single-exponential functions with time constants of 761 ± 48 ms and 187 ± 12 ms ($n = 9$), respectively (Fig. 5A). The stationary level of the current induced by 3-s ASP and DTT coapplication, I_{CP}, was 2.03 ± 0.20 ($n = 15$) times larger than that of control, I_C (Fig. 5B). EOS did not affect DTT potentiation. Thus, DTT increased the ASP-induced current with equal effectiveness both in the absence (2.28 ± 0.12, $n = 4$) and presence (2.28 ± 0.26, $n = 4$) of 222 μM EOS (these values were not significantly different, $P > .99$). The degree of the EOS (222 μM)-induced stationary current inhibition was the same in the presence, $I_{BP}/I_{CP} = 0.45 \pm 0.05$, and absence, $I_B/I_C = 0.45 \pm 0.07$, of DTT, respectively (these values were not significantly different, $P > .9$, $n = 4$) (Fig. 5B). EOS inhibited DTT-potentiated currents in a concentration-dependent manner (Fig. 5C). The fitting of the I_{BP}/I_{CP} dependence with the logistic equation gave the following values: $IC_{50} = 266 \pm 32$ μM; $n_H = 1.28 \pm 0.15$ ($n = 5$), which did not differ significantly from those of control (Fig. 2B).

Another reducing agent, glutathione (1 mM), exhibited an analogous, but much weaker in comparison with DTT, effect on NMDA-mediated currents. Its effect was also fast. The onset and offset kinetics of glutathione-induced potentiation were well fitted with single-exponential functions with the time constants of 288 ± 84 and 197 ± 31 ms ($n = 3$), respectively. The stationary level of the glutathione-potentiated current was only 1.22 ± 0.11 ($n = 6$) times higher than that of control. As in the case of DTT, the degree of the EOS (222 μM)-induced stationary current inhibition was the same in the presence, $I_{BP}/I_{CP} = 0.41 \pm 0.04$, and absence, $I_B/I_C = 0.44 \pm 0.05$, of glutathione, respectively (these values were not significantly different, $P > .4$, $n = 6$).

To find out to what extent potentiation produced by reducing agents resulted from chelation of heavy metals (Paoletti et al., 1997), we carried out the experiments with EDTA. In 8 of 11 cells, EDTA (10 μM) potentiated the NMDA currents. The kinetics of this potentiation was studied according to the experimental protocol shown in Figs. 2C and 5A. The onset and offset kinetics of EDTA-induced potentiation were well fitted with single-exponential functions with time constants of 2.03 ± 0.19 s and 216 ± 23 ms ($n = 5$), respectively. When coapplied for 10 s with ASP (100 μM), EDTA (10 μM) potentiated the stationary current much weaker than DTT (Fig. 6). Thus, the I_{CP}/I_C value for DTT (1.85 ± 0.07) was significantly ($P < 10^{-5}$, $n = 10$) higher than that for EDTA (1.23 ± 0.05). This indicates that DTT-induced potentiation can only partly be explained by heavy metal chelation. However, it is unlikely that such chelation was responsible for nonadditive potentiations produced by EDTA and DTT (Fig. 6). Thus, the I_{CP}/I_C value for DTT plus EDTA coapplication (1.73 ± 0.06, $n = 10$) was lower ($P < .001$) than that for DTT alone (1.85 ± 0.07, $n = 10$).

Therefore, in our experiments the reducing agents acted mainly at the redox site and not via chelation of Zn^{2+} as proposed by Paoletti et al. (1997). Correspondingly, the fact that DTT and glutathione did not alter significantly the

blocking action of EOS most probably implies that EOS does not interact with the NMDA channel redox site.

To elucidate whether EOS competes with protons for common binding sites, we studied EOS-induced inhibition of NMDA responses at low external pH. Protons equally well inhibited NMDA-mediated currents both in the absence and
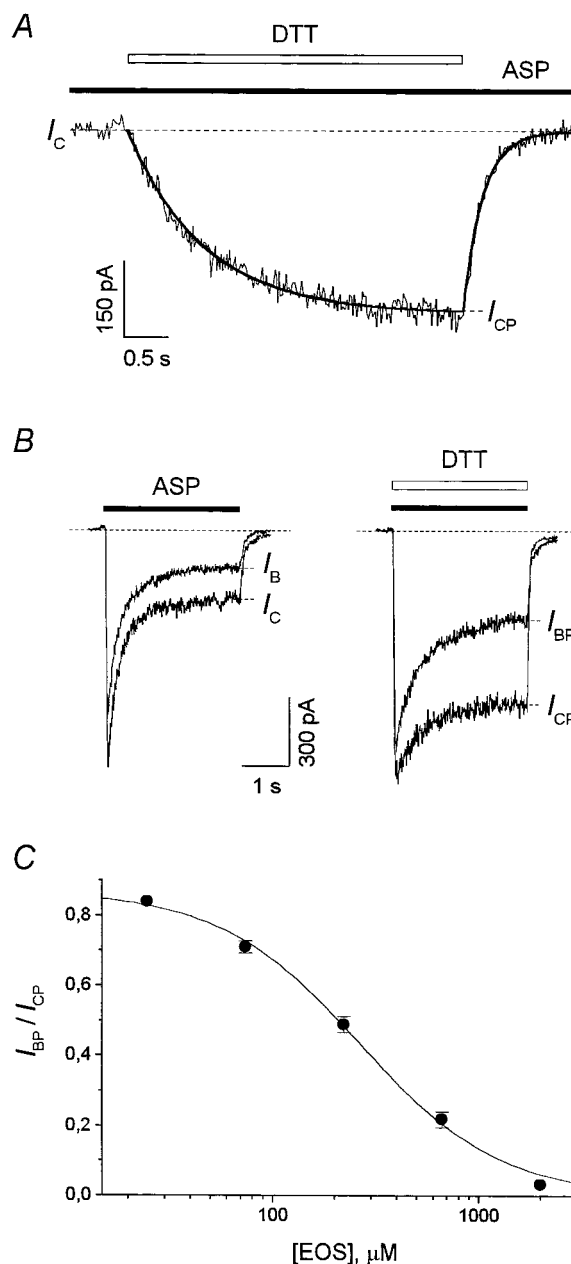

Fig. 5. The effects of DTT and EOS on NMDA responses. A, DTT (3 mM) when applied in the continuous presence of ASP (100 μM) after the current reached its stationary level, I_C, potentiated the NMDA responses. The onset and offset kinetics of the DTT-induced potentiation were fitted with single-exponential functions (solid lines) with time constants of 842 and 224 ms, respectively. B, current responses elicited by ASP and ASP plus 222 μM EOS (left) as well as by ASP and ASP plus EOS coapplied with 3 mM DTT (right) are superimposed. The degree of EOS-induced stationary current inhibition was the same in the absence, I_B/I_C, and presence, I_{BP}/I_{CP}, of DTT. C, concentration dependence of the EOS-induced stationary current inhibition measured in the presence of DTT (3 mM) as shown in B (right). The I_{BP}/I_{CP} dependence was fitted with the logistic equation (solid line). The fit parameters were as follows: $IC_{50} = 266 \pm 32$ μM and $n_H = 1.28 \pm 0.15$ ($n = 5$).

presence of EOS. Thus, the stationary value of I(pH 7.3)/I(pH 6.8) was practically the same in the absence (2.26 $\pm$ 0.20) and presence (2.33 $\pm$ 0.23) of 222 μM EOS (these values were not significantly different, $P > .7$, $n = 6$). Although the stationary control current at pH = 6.8 was smaller than at pH = 7.3, EOS-induced stationary current inhibition was the same both at pH = 7.3 ($I_B/I_C = 0.46 \pm 0.03$) and at pH = 6.8 ($I_B/I_C = 0.45 \pm 0.03$) (these values were not significantly different, $P > .9$, $n = 6$) (Fig. 7A).

Then, we studied the concentration dependence of the EOS-induced stationary current inhibition at pH = 6.8 (Fig. 7B). The fitting of the I_B/I_C curve at pH = 6.8 with the logistic equation gave the following values: $IC_{50} = 286 \pm 21$ μM and $n_H = 1.43 \pm 0.12$ ($n = 6$), which did not differ significantly from those at pH = 7.3 (Fig. 2B). Thus, EOS-induced inhibition of NMDA responses at pH = 7.3 and pH = 6.8 was equally effective. This most probably implies that the EOS and proton binding sites do not overlap.

In the next series of experiments, we tested the possibility of EOS interaction with Zn^{2+} binding site. Figure 8A shows an example of currents induced by ASP and ASP plus 11 μM

Zn^{2+} applications in the absence (left traces) and presence (right traces) of 200 μM EOS. Despite the fact that the stationary current was greatly suppressed by EOS, Zn^{2+} inhibited it with equal efficiency both in the absence ($I_B/I_C = 0.38 \pm 0.02$) and presence ($I_B/I_C = 0.37 \pm 0.02$) of EOS (these values were not significantly different, $P > .6$, $n = 6$). To find out whether EOS affected the affinity of Zn^{2+} for NMDA channels, we studied the concentration dependencies of Zn^{2+}-induced inhibition of the stationary current in the presence and absence of EOS. Figure 8B shows the values of the stationary currents in the presence and absence of EOS divided by the control current, I_C, at different Zn^{2+} concentrations. The fitting of the dose-effect relation by a logistic equation yielded the following values: $IC_{50} = 7.3 \pm 1.3$ μM, $n_H = 0.89 \pm 0.08$ ($n = 6$) in the absence and $IC_{50} = 8.7 \pm 0.6$ μM, $n_H = 1.22 \pm 0.07$ ($n = 6$) in the presence of EOS. Thus, Zn^{2+} inhibited NMDA responses equally well in the presence and absence of EOS. This most probably means that EOS does not compete with Zn^{2+} for common binding sites.

EOS-induced inhibition did not change when NMDA currents were potentiated by the polyamine spermine (Fig. 9A). When coapplied with ASP, 500 μM spermine elicited currents 71 $\pm$ 9% ($n = 6$) greater than control. Nevertheless, the EOS (222 μM)-induced stationary current inhibition was practically the same both in the presence ($I_B/I_C = 0.50 \pm 0.02$) and absence ($I_B/I_C = 0.49 \pm 0.02$) of spermine (these values were not significantly different, $P > .7$, $n = 6$).
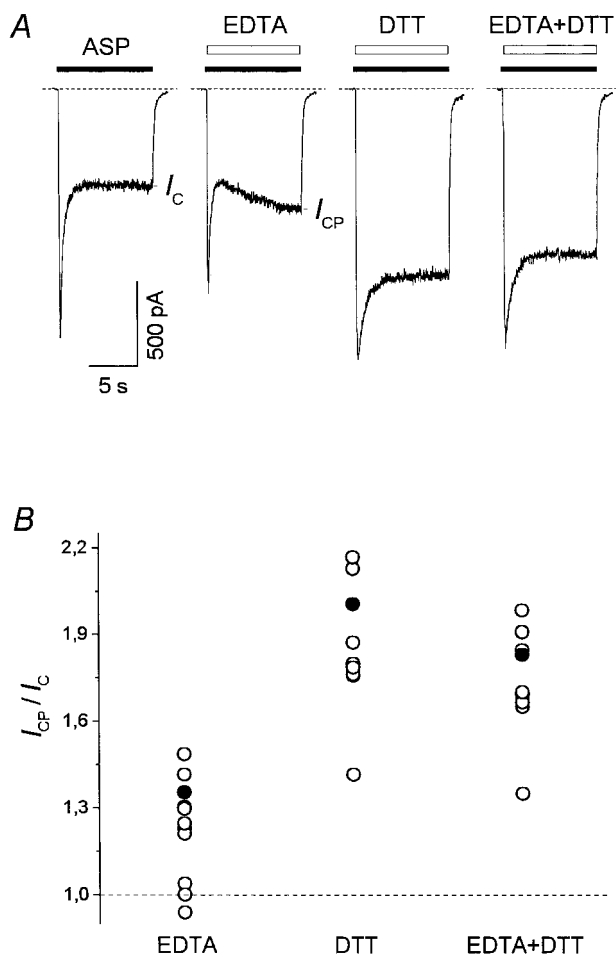

Fig. 6. DTT-induced potentiation is only partly caused by chelation of contaminant heavy metals. A, NMDA currents induced by 10-s ASP (100 μM) application and ASP coapplication with EDTA (10 μM), DTT (3 mM), and EDTA (10 μM) plus DTT (3 mM). DTT induced much stronger potentiation than EDTA. B, comparison of effects of EDTA and DTT on the stationary current inhibition. Each circle corresponds to the I_{CP}/I_C value obtained from one experiment. ● corresponds to the experiment illustrated in A.
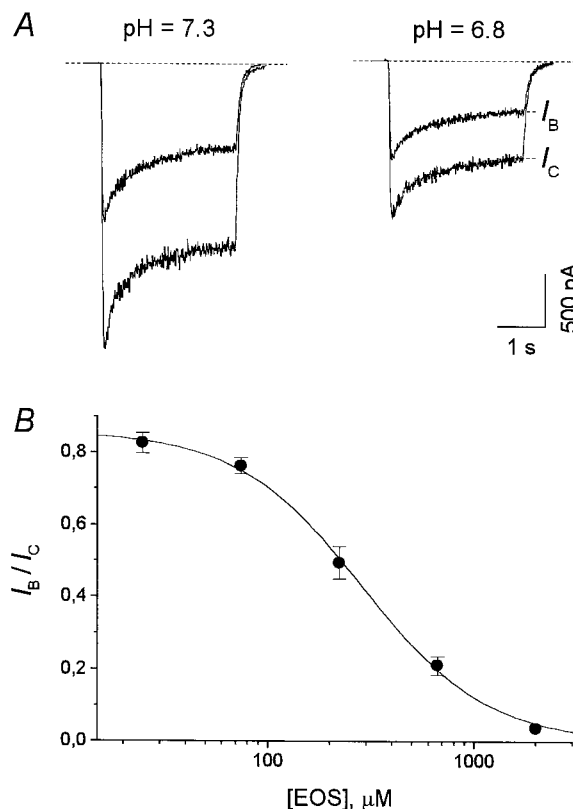
Fig. 7. EOS-induced inhibition of NMDA responses did not depend on extracellular pH. A, superposition of currents in response to ASP and ASP plus 222 μM EOS at pH = 7.3 (left) and pH = 6.8 (right). The degree of the stationary current inhibition by 222 μM EOS was approximately the same at different pH. B, dependence of the stationary current inhibition at pH = 6.8 on EOS concentration. The fitting of the I_B/I_C curve with the logistic equation (solid line) yielded the following values of parameters: $IC_{50} = 286 \pm 21$ μM and $n_H = 1.43 \pm 0.12$ ($n = 6$).

Finally, we checked the ability of EOS to compete with ethanol (Fig. 9B). Ethanol (100 mM) induced $33 \pm 3\%$ ($n = 8$) inhibition of the NMDA-mediated current. However, the EOS (222 μM) block was equally effective both in the presence ($I_B/I_C = 0.53 \pm 0.03$) and absence ($I_B/I_C = 0.52 \pm 0.05$) of ethanol (these values were not significantly different, $P > .7$, $n = 8$).

Discussion

This study shows for the first time that EOS inhibits ionic currents through NMDA channels in a time- and concentration-dependent manner (Fig. 2). The incomplete recovery from inhibition at concentrations higher than 1 mM (Fig. 2A) possibly resulted from nonspecific action of EOS, and this question demands an additional study.

The EOS-induced inhibition proved to be voltage-independent (Fig. 3). This fact led us to check whether EOS competes with the agonist or the coagonist for binding sites on the NMDA receptor. A series of NMDA antagonists was shown to

interact with NMDA (Watkins and Olverman, 1987; Benveniste and Mayer, 1991, 1992) and GLY (Benveniste et al., 1990; Kemp and Priestley, 1991; Guzikowski et al., 1996; Parsons et al., 1997; Honer et al., 1998) binding sites. In the case of EOS, the ASP and GLY independence (Fig. 4) clearly demonstrate a noncompetitive mechanism of its block.

There are several NMDA receptor modulators whose action does not depend on the membrane potential. The first group of such modulators includes the reducing/oxidizing agents (for reviews see Lipton, 1993; McBain and Mayer, 1994; Dingledine et al., 1999). Thus, DTT and glutathione were found to potentiate NMDA responses (Aizenman et al., 1989; Kohr et al., 1994). In our experiments, the DTT- and glutathione-induced potentiation did not significantly alter the EOS-induced block (Fig. 5). To find out whether potentiation by reducing agents was due to their chelation of contaminant heavy metals (Paoletti et al., 1997), we performed experiments with EDTA (Fig. 6). The results of these experiments allowed the conclusion that reducing agents interacted mainly with the putative redox regulatory site on NMDA receptor and not with heavy metal binding sites.

In experiments with NR1-NR2A receptors, Paoletti et al. (1997) showed that dithioerythritol, the erythroisomer of DTT, failed to induce fast potentiation of NMDA currents in the presence of EDTA. However, in our experiments, DTT potentiated ASP-induced currents even in the presence of EDTA. Potentiation of NR1-NR2A receptors by heavy metal chelators in the study of Paoletti et al. (1997) was 1.6- to 1.7-fold and did not depend on type of the chelator, whereas NR1-NR2B receptors were not potentiated by heavy metal
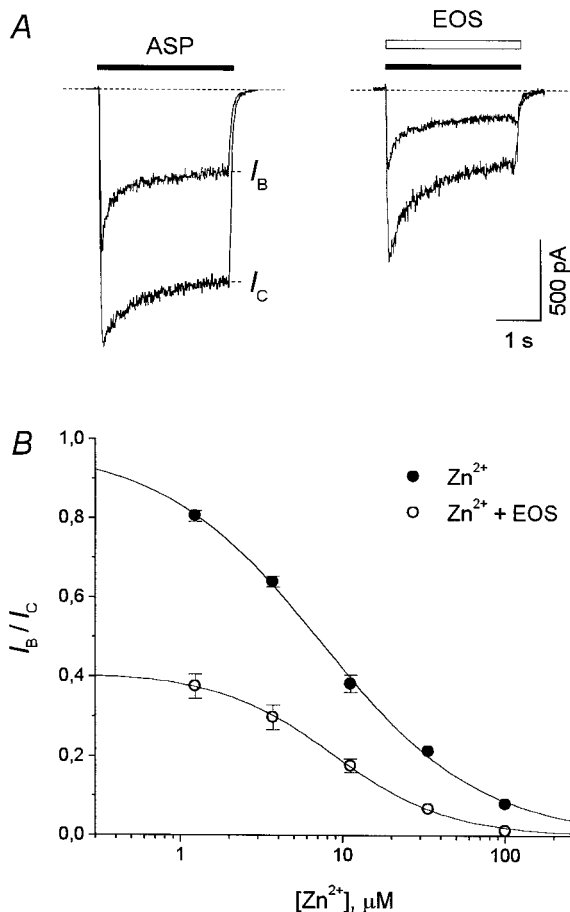

Fig. 8. Zn^{2+} inhibited NMDA responses with equal efficiency both in the presence and absence of 200 μM EOS. A, current responses elicited by ASP and ASP plus 11 μM Zn^{2+} (left) as well as by ASP and ASP plus Zn^{2+} coapplied with 200 μM EOS (right) are superimposed. The degree of Zn^{2+}-induced stationary current inhibition was the same in the absence and presence of EOS. B, dependence of the values of the stationary current inhibited by Zn^{2+} in the presence and absence of 222 μM EOS divided by the control current value, I_C, on Zn^{2+} concentration. The parameters of the fitting of the concentration dependencies with the logistic equation (solid lines) are as follows: $IC_{50} = 7.3 \pm 1.3$ μM, $n_H = 0.89 \pm 0.08$ ($n = 6$) in the absence and $IC_{50} = 8.7 \pm 0.6$ μM, $n_H = 1.22 \pm 0.07$ ($n = 6$) in the presence of EOS.
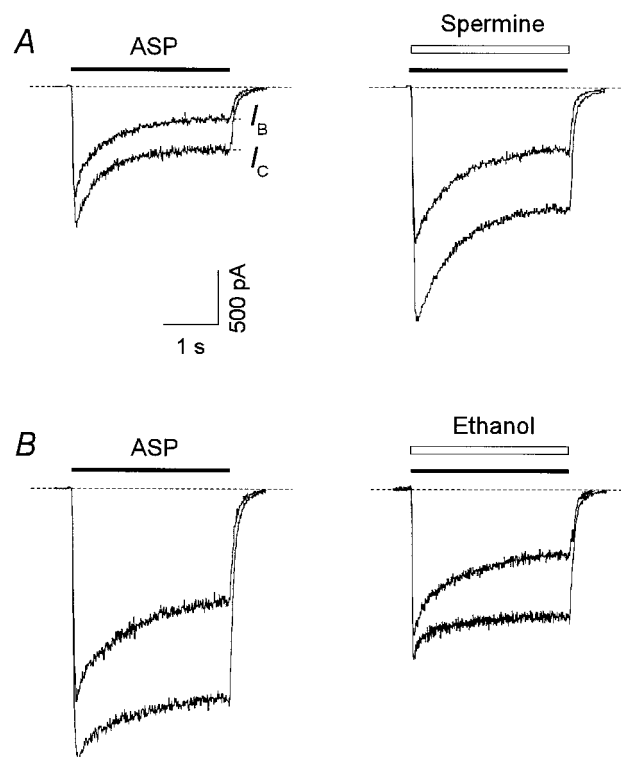

Fig. 9. Spermine and ethanol did not affect the EOS (222 μM)-induced inhibition of NMDA-mediated currents. ASP was coapplied for 3 s with different drugs. A, superposition of the control and EOS-inhibited currents in the absence (left) and presence (right) of 500 μM spermine. B, same superposition in the absence (left) and presence (right) of 100 mM ethanol.

chelators at all. In our study, potentiation of the ASP-induced currents by EDTA was only 1.2, and in 3 of 11 cells was absent. All of these findings suggest that the acutely isolated rat hippocampal neurons mainly express the NR1-NR2B receptors or these receptors together with some number of NR1-NR2A receptors (or probably NR1-NR2A-NR2B receptors). However, in contrast to recombinant NR1-NR2B receptors (Kohr et al., 1994), the DTT-induced potentiation in acutely isolated rat hippocampal neurons is rather fast and reversible (Fig. 5).

The ability of Zn^{2+} as well as of other heavy metals (Cd^{2+}, Fe^{2+}, and Cu^{2+}) to effectively inhibit NMDA responses in a noncompetitive and voltage-independent manner was shown in many previous studies (Peters et al., 1987; Westbrook and Mayer, 1987; Mayer et al., 1988, 1989; Christine and Choi, 1990; Legendre and Westbrook, 1990; Eimerl and Schramm, 1993; Trombley and Shepherd, 1996; Vlachova et al., 1996; Paoletti et al., 1997). According to our experiments with 10 μM EDTA (Fig. 6), there was some contamination of the external solution by heavy metals. However, EDTA induced only weak potentiation of NMDA currents. Moreover, in 3 of 10 cells this potentiation was absent. This indicates that in some cells there were no NMDA receptors with high-affinity heavy metal blocking sites. In contrast, EOS blockade was observed in all cells. This fact along with experiments illustrated in Fig. 8 most probably means that the EOS effect is mediated by a site different from heavy metal inhibitory sites.

There was previously reports that NMDA responses can be inhibited in a voltage-independent manner by protons (Tang et al., 1990; Traynelis and Cull-Candy, 1990, 1991; Vyklicky et al., 1990), polyamines (McGurk et al., 1990; Rock and MacDonald, 1992; Benveniste and Mayer, 1993), and alcohols (Chu et al., 1995; Peoples et al., 1997; Peoples and Weight, 1998). However, our experiments (Figs. 7 and 9) indicate that EOS and either of these regulators have different targets on NMDA receptor channel.

Voltage-independent inhibition of NR1A/NR2B receptors was reported for ifenprodil (Williams, 1993). However, this effect of ifenprodil was reduced by increasing the concentration of GLY (Williams, 1993). Moreover, Mott et al. (1998) showed that ifenprodil increases the ability of protons to block the NMDA receptor. Taking into account the independence of the EOS-induced blockade of NMDA channels on both GLY and pH (Figs. 4C and 7), we predict that the effects of ifenprodil and EOS are most probably mediated via different NMDA receptor regulatory sites.

Another noncompetitive NMDA receptor antagonist that can act at the same inhibitory site as EOS is the opioid peptide dynorphin (Chen et al., 1995). However, the block by dynorphin was shown to be substantially weakened by DTT. In contrast, the EOS-induced inhibition of NMDA-mediated currents proved to be DTT independent (Fig. 5). Thus, we predict that EOS and dynorphin do not share common binding sites on the NMDA receptor channel.

Therefore, we believe that EOS acts at a new, previously undescribed, NMDA receptor inhibitory site. This site can overlap with sites of *n*-alkyl diamines or KB-R7943, where blockade of NMDA channels was shown to be partly voltage independent (Subramaniam et al., 1994; Sobolevsky and Khodorov, 1999).

The EOS-induced blockade of NMDA channels in neuronal cells should be taken into account when EOS is used as a specific inhibitor of the plasma membrane Ca^{2+} pump. The binding site mediating this blocking effect of EOS is located intracellularly. However, EOS does not readily cross the plasma membrane. Therefore, in the case of its external application to the intact cell, effective EOS-induced blockade of the Ca^{2+} pump can only be obtained by using it at concentrations exceeding those required to block the Ca^{2+} pump from inside the cell by several hundred fold (Gatto et al., 1995). This can be considered as one of the serious limitations for the use of EOS as a tool to study Ca^{2+} homeostasis in various cells. The data obtained indicate that along with an inhibitory action on the Ca^{2+} pump, the ability of EOS to block NMDA channels should be taken into consideration in studies of EOS effects on intact nerve cells.

It seems likely that EOS could potentially act nonselectively to inhibit the activity of other membrane proteins due to its membrane solubility at the high concentrations needed. To find out the selectivity of EOS action on NMDA receptors, additional studies should be performed to exclude its action on Na^+, K^+, and Ca^{2+} channels as well as on other neurotransmitter receptor channels.

In conclusion, the properties of EOS-induced inhibition point to the existence of a new, previously undescribed, NMDA receptor regulatory site. This fact indicates that EOS can be a tool for studying the structure and function of NMDA receptors. The existence of the new regulatory site may imply the existence of unknown endogenous modulators of NMDA receptor functions. The discovery of the new NMDA receptor regulatory site is important not only for understanding the regulation of this receptor more fully; this discovery also provides a new target for high-affinity EOS analogs that could possibly be applicable in medical practice for the treatment of neurological disorders.

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