**Direct Inhibition of the N-methyl-D-aspartate Receptor Channel by High Concentrations of Opioids**

Tomohiro Yamakura, M.D.,* Kenji Sakimura, Ph.D.,† Koki Shimoji, M.D.‡

**Background:** Electrophysiologic and receptor binding studies showed that some opioids have noncompetitive N-methyl-D-aspartate (NMDA) receptor antagonist properties.

**Methods:** The effects and mechanisms of action of various opioid compounds were examined on four kinds of heteromeric NMDA receptor channels, namely the e1/z1, e2/z1, e3/z1, and e4/z1 channels, expressed in *Xenopus* oocytes. Furthermore, the action sites of opioids on NMDA receptor channels were investigated by site-directed mutagenesis.

**Results:** Meperidine inhibited four kinds of channels to a similar extent with inhibitor concentrations for half-control response (IC50s) of 210–270 μM. Morphine, fentanyl, codeine, and naloxone also inhibited NMDA receptor channels with affinities comparable to meperidine. Opioid inhibition exhibited voltage dependence and was quite effective at negative potentials. Opioids also shifted the inhibition curve of Mg2+ to the right. Furthermore, replacement of the conserved asparagine residue with glutamine in the channel-lining segment M2 of the z1 subunit, which constitutes the block sites of Mg2+ and ketamine, reduced the sensitivity to opioids, whereas that of the e2 subunit barely affected the opioid sensitivity.

**Conclusions:** These results, together with previous findings, suggest that the low-affinity NMDA receptor antagonist activity is a common characteristic of various opioid compounds, and that the inhibition is a result of channel-block mechanisms at the site, which partially overlaps with those of Mg2+ and ketamine. This antagonist property of opioids may be clinically significant in the spinal cord following epidural or intrathecal administration, after which the cerebrospinal fluid concentrations of some opioids reach the high micromolar level. (Key words: Channel blocker; dissociative anesthetics; glutamate receptor; subunit.)

* Instructor, Department of Anesthesiology.
† Professor, Department of Cellular Neurobiology, Brain Research Institute.
‡ Professor and Chairman, Department of Anesthesiology.

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Address reprint requests to Dr. Yamakura: Department of Anesthesiology, Niigata University School of Medicine, 1-757 Asahimachi, Niigata 951-8510, Japan. Address electronic mail to: yamakura@med.niigata-u.ac.jp
NMDA receptor channel subunits have four hydrophobic segments (M1–M4) within their central regions. According to the three transmembrane segment model, segment M2 forms a reentrant membrane loop with both ends facing the cytoplasm, and the carboxyl-terminal region resides in the cytoplasm (fig. 1A). Site-directed mutagenesis has revealed that the conserved asparagine (N) residues in segment M2 of the ε and ζ subunits govern both Mg²⁺ block and Ca²⁺ permeability of NMDA receptor channels, thus indicating that segment M2 constitutes the ion-channel pore of NMDA receptor channels. The position of this asparagine residue corresponds to that of glutamine (Q) or arginine (R) of the α subunits, which determines the Ca²⁺ permeability of the AMPA-selective glutamate receptor channel.

In the present investigation, we examined the effects and mechanisms of action of various opioid compounds (phenylpiperidine derivatives meperidine and fentanyl, and opioid antagonist naloxone) on the naturally occurring opioids morphine and codeine, and those selective for AMPA (α1, α2, α3, and α4). The box indicates the Q (glutamine)/R (arginine)/N (asparagine) site of the glutamate receptor channel subunits, which determines the permeability and block by divergent cations.

**Materials and Methods**

**Subunit-specific mRNA Preparation and Expression in Xenopus Oocytes**

Subunit-specific mRNAs were synthesized in vitro with SP6 or T3 RNA polymerase (Ambion MEGAscript) in the presence of cap dinucleotides 5’GpppG. The ε1, ε2, ε3, ε4, and ζ1 subunit-specific mRNAs (for the expression of the ε1/ζ1, ε2/ζ1, ε3/ζ1, and ε4/ζ1 channels) were synthesized using pSPGRε1, pSPGRε2, pSPGRε3, and pSPGRε4, respectively. The ε2, ε2-N589Q, and ε2/ζ1-N589Q subunit-specific mRNAs (for the expression of the ε2-N589Q/ζ1, ε2/ζ1-N589Q, and ε2-N589Q/ε2-N589Q channels) were synthesized using pBKSAe2, pBKSAe2-N589Q, and pBKSAe2/ζ1-N589Q, respectively. The α1 and α2 subunit-specific mRNAs for the α1/α2 AMPA-selective glutamate receptor channel were synthesized using pSPGR1 and pSPGR2, respectively.

*Xenopus laevis* oocytes were injected with the wild-type or mutant ε subunit-specific mRNA and the wild-type or mutant ζ subunit-specific mRNA at a molar ratio of 1:1, or with the α1 and α2 subunit-specific mRNAs at a molar ratio of 10:1; the total amount of mRNAs injected per oocyte was approximately 0.6 ng for the ε1/ζ1 and ε2/ζ1 channels; 14 ng for the ε3/ζ1 and ε4/ζ1 channels; 4 ng for the ε2-N589Q/ζ1, ε2/ζ1-N589Q, and ε2-N589Q/ε2-N589Q channels; and 10 ng for the α1/α2 channel.

**Electrophysiological Analyses**

After incubation at approximately 19°C for 2 or 3 days, whole-cell currents evoked by bath application of agonists for approximately 15 s were recorded at −70 mV membrane potential with a conventional two-micro pipette voltage clamp. The current responses of the wild-type and mutant ε/ζ channels to 10 μM 1-glutamate plus 10 μM glycine (almost saturating concentrations for all ε/ζ channels) were measured in Ba²⁺-Ringer’s solution to minimize the effects of secondarily activated Ca²⁺-dependent CI currents. The current responses of the α1/α2 channel to 100 μM kainate were measured in normal frog Ringer’s solution. For measurement of the...
effects of opioids on NMDA receptor channels, opioids were continuously perfused during the experiment. Pre-application of opioids in the absence of agonists did not produce any current response in either wild-type or mutant channels. Agonists were applied three times successively during perfusion of opioids, and the effects on the second and third applications of agonists were averaged. The second and third current responses during perfusion of opioids were of similar magnitude, indicating that the effects of opioids were fully established in this recording system. Ba^{2+}-Ringer’s solution contained 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, and 10 mM HEPES-NaOH (pH 7.2). Normal frog Ringer’s solution contained 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, and 10 mM HEPES-NaOH (pH 7.2).

Compounds
Meperidine hydrochloride was purchased from Tanabe Seiyaku (Osaka, Japan). Morphine hydrochloride, codeine phosphate, and fentanyl citrate were from Sankyo (Tokyo, Japan). Naloxone hydrochloride was from Sigma Chemical (St. Louis, MO). Meperidine, morphine, codeine, and naloxone were dissolved in distilled water at a concentration of 100 mM. Fentanyl was dissolved in dimethyl sulfoxide at a concentration of 100 mM. The dimethyl sulfoxide stocks were diluted to appropriate concentrations in Ringer’s solution. Perfusion of the highest concentrations of dimethyl sulfoxide used in this investigation (1% for 1 mM fentanyl) inhibited the current responses of the e2/ξ1 channel by 5 ± 1% (mean ± SEM, n = 4).

Statistical Analysis
The inhibitor concentration for half-control response (IC₅₀) and the Hill coefficient values for opioids of the e/ξ channel were calculated according to the equation
\[ Ropi = 1/[1 + (O/IC₅₀)^n] \]
where \( Ropi \) represents the relative response, \( O \) the concentration of opioids, and \( n \) the Hill coefficient. The agonist concentration for half-control response (EC₅₀) of the e/ξ channel was calculated according to the equation
\[ Rago = Fopi/[1 + (EC₅₀/A)^n] \]
where \( Rago \) represents the relative response, \( Fopi \) the residual fraction by opioid inhibition of responses to saturating concentrations of agonists, \( A \) the concentration of agonists, and \( n \) the Hill coefficient. For quantitative estimates of the voltage dependence of block by opioids, data were analyzed using the Woodhull model by fitting the data to the equation
\[ Ropi = 1/[1 + (O/K_{d0} \exp(z\delta E/RT))] \]
where \( Ropi \) represents the relative response, \( O \) the concentration of opioids, \( K_{d0} \) the equilibrium dissociation constant of opioids at a membrane potential of 0 mV, \( z \) the charge of opioids, \( \delta \) the portion of the membrane electric field sensed at the blocking site, \( E \) the membrane potential, \( F \) the Faraday constant, \( R \) the gas constant, and \( T \) the absolute temperature. The results obtained were statistically analyzed by the Student t test or one-way analysis of variance (ANOVA) followed by Scheffé’s multiple comparison tests. \( P < 0.05 \) was considered significant. Data were represented as mean ± SEM.

Results

Effects of Opioids on Four Kinds of e/ξ Heteromeric NMDA Receptor Channels
Four kinds of heteromeric NMDA receptor channels, the e1/ξ1, e2/ξ1, e3/ξ1, and e4/ξ1 channels, were expressed in Xenopus oocytes by the injection of respective subunit-specific mRNAs synthesized in vitro from cloned cDNAs. The sensitivities of these e/ξ heteromeric channels to opioids were examined by measuring current responses to 10 μM L-glutamate plus 10 μM glycine during continuous perfusion of meperidine at −70 mV membrane potential in Ba^{2+}-Ringer’s solution. Meperidine inhibited the current responses of the e/ξ NMDA receptor channels (fig. 2A). After the meperidine was washed out, application of agonists two or three times fully recovered the current responses. The dose-inhibition relationships for meperidine of four kinds of heteromeric channels were examined (fig. 2B). Meperidine inhibited the e1/ξ1, e2/ξ1, e3/ξ1, and e4/ξ1 channels to a similar extent in a concentration-dependent manner. The IC₅₀ values (μM) of the e1/ξ1, e2/ξ1, e3/ξ1, and e4/ξ1 channels for meperidine were 233 ± 4 (n = 6), 206 ± 7 (n = 7), 264 ± 9 (n = 6), and 273 ± 12 (n = 6), respectively. The e2/ξ1 channel was more sensitive to meperidine than the e3/ξ1 and e4/ξ1 channels (log[IC₅₀] values were compared by ANOVA followed by Scheffé’s multiple comparison tests; \( P < 0.01 \)). On the other hand, 1 mM meperidine only inhibited the current responses of the α1/α2 glutamate receptor channel selective for AMPA by 6 ± 3% (n = 5). Thus, the inhibitory effects of meperidine are likely to be selective for NMDA receptor channels out of the glutamate receptor channels. Because meperidine inhibited NMDA receptor channels, the effects of other opioid agonists and antagonists on NMDA receptor channels were examined. Morphine also inhibited the four e/ξ channels in...
a dose-dependent manner. The sensitivities to morphine varied between the four $e/\zeta$ channels (log[IC$_{50}$] values were compared by ANOVA; $P < 0.0001$; fig. 2C). The IC$_{50}$ values ($\mu$M) of the $e1/\zeta1$, $e2/\zeta1$, $e3/\zeta1$, and $e4/\zeta1$ channels were $321 \pm 48$ ($n = 7$), $187 \pm 9$ ($n = 7$), $392 \pm 27$ ($n = 6$) and $650 \pm 24$ ($n = 7$), respectively. The $e2/\zeta1$ channel was the most sensitive to morphine among the four $e/\zeta$ channels (Scheffé’s multiple comparison tests, $P < 0.05$). In addition, fentanyl, codeine, and naloxone also inhibited NMDA receptor channels in a dose-dependent manner (fig. 2D). The IC$_{50}$ values ($\mu$M) of the $e2/\zeta1$ channel for fentanyl, codeine, and naloxone were $192 \pm 9$ ($n = 7$), $613 \pm 25$ ($n = 7$), and $503 \pm 34$ ($n = 7$), respectively.
Effects of Opioids on the Dose-Response Relationships of the NMDA Receptor Channel with Agonists

To characterize the inhibitory effects of opioids on NMDA receptor channels, we examined whether opioids affect the apparent affinities of the e2/ζ1 channel for agonists. The dose–response relationships of the e2/ζ1 channel for L-glutamate and glycine before and during perfusion of 300 μM meperidine were analyzed (fig. 3A). Meperidine effectively suppressed the maximal current responses to saturating concentrations of both L-glutamate and glycine. The EC50 values (μM) of the e2/ζ1 channel for L-glutamate in the presence of 10 μM glycine and those for glycine in the presence of 10 μM L-glutamate during perfusion of 300 μM meperidine (1.04 ± 0.04 [n = 7] and 0.29 ± 0.01 [n = 6], respectively) were not significantly different from those before meperidine perfusion (1.03 ± 0.02 [n = 6] and 0.29 ± 0.01 [n = 6], respectively) (log[EC50] values were compared by t tests; P > 0.76 for L-glutamate and P > 0.81 for glycine). Similarly, morphine (300 μM) inhibited the maximal current responses of the e2/ζ1 channel without affecting the EC50 values (fig. 3B). These results suggest the non-competitive antagonism of NMDA receptor channels by opioids.

Effects of the Membrane Potential on Opioid Inhibition

To test whether the inhibition by opioids is voltage-dependent, the extent of inhibition was measured at different holding potentials. Figure 4A shows current-voltage relationships of the e2/ζ1 channel before and during perfusion of 300 μM meperidine. Meperidine inhibition of the e2/ζ1 channel exhibited voltage dependence and was quite effective at hyperpolarized potentials. The extent of inhibition was significantly dependent on the membrane potential (ANOVA, P < 0.0001). At a membrane potential of −110 mV, meperidine (300 μM) reduced the current responses of the e2/ζ1 channel to 17 ± 1% (n = 7) of the control responses, whereas at −10 mV membrane potential, meperidine reduced the current responses to only 85 ± 2% (n = 7). Similarly, morphine (300 μM) inhibited the e2/ζ1 channel in a voltage-dependent manner (fig. 4B).

The degree of voltage dependence of inhibition of the e2/ζ1 channel by meperidine (300 μM), morphine (300 μM), and naloxone (1 μM) was compared using the Woodhull model 21 (fig. 4C). Although the Kd(0) values (the affinity of binding) for meperidine, morphine, and naloxone varied (2.6 ± 0.5 [n = 7], 3.8 ± 0.5 [n = 8], and 10.7 ± 1.6 [n = 8] mM, respectively; ANOVA, P < 0.0001), the zδ values (the degree of voltage dependence of block) for those were not significantly different.
Effects of Opioids on the Sensitivities to Mg\(^{2+}\) Block

To determine whether opioids interact with the Mg\(^{2+}\) block site of NMDA receptor channels, we examined the effects of meperidine (300 \(\mu\)M) and morphine (300 \(\mu\)M) on the sensitivity of the \(\varepsilon_2/\zeta_1\) channel to the Mg\(^{2+}\) block (fig. 5A). Mg\(^{2+}\) inhibited the current responses of the \(\varepsilon_2/\zeta_1\) channel in a dose-dependent manner with IC\(_{50}\) values of 15.5 \(\pm\) 1.0 \(\mu\)M (n = 8). Meperidine and morphine shifted the inhibition curve of Mg\(^{2+}\) to the right (fig. 5B). The IC\(_{50}\) values (\(\mu\)M) for Mg\(^{2+}\) during perfusion of meperidine and morphine were 30.0 \(\pm\) 1.9 (n = 7) and 37.0 \(\pm\) 2.5 (n = 7), respectively, which were significantly higher than those for Mg\(^{2+}\) alone (log[IC\(_{50}\)] values were compared using ANOVA followed by Scheffe’s multiple comparison tests, \(P < 0.0001\) for control versus meperidine, and control versus morphine).

Effects of Point Mutations on Inhibition by Opioids

Opioids inhibited the current responses of the NMDA receptor channel in a voltage-dependent manner. The voltage-dependent inhibition is a specific and essential property of the well-characterized NMDA receptor channel blockers, such as Mg\(^{2+}\) and dissociative anesthetics (phencyclidine [PCP], ketamine, and (+)-MK-801).\(^{22,23}\) Furthermore, the Mg\(^{2+}\) block curve was shifted rightward by opioids. These results suggest that inhibition of NMDA receptor channels by opioids may be a result of channel block mechanisms. We have previously shown that the conserved asparagine residue in the channel-lining segment M2 of the \(\varepsilon_2\) and \(\zeta_1\) subunits (the asparagine 589 of the \(\varepsilon_2\) subunit and the asparagine 598 of the \(\zeta_1\) subunits) constitutes the Mg\(^{2+}\) block site of NMDA receptor channels, and that the noncompetitive antagonists, PCP, ketamine, \(n\)-allylnormetazocine (SKF-10,047), and (+)-MK-801, also act on the Mg\(^{2+}\) block site.\(^{11,13}\) To reveal whether the same asparagine residue also constitutes the block site of opioids, we examined the effects of replacement by glutamine of the conserved asparagine residue in segment M2 of the \(\varepsilon_2\) and \(\zeta_1\) subunits (the mutations \(\varepsilon_2\)-N589Q and \(\zeta_1\)-N598Q, respectively) on the sensitivity to opioids. The mutation \(\zeta_1\)-N598Q reduced the sensitivity to meperidine (fig. 6A). The \(\varepsilon_2/\zeta_1\)-N598Q and \(\varepsilon_2\)-N589Q/\(\zeta_1\)-N598Q channels were more resistant to meperidine than the \(\varepsilon_2/\zeta_1\) and \(\varepsilon_2\)-N589Q/\(\zeta_1\) channels (log[IC\(_{50}\)] values were compared by ANOVA followed by Scheffe’s multiple comparison tests, \(P < 0.0001\)) (fig. 6B). On the other hand, the sensitivity of

Fig. 4. The effects of the membrane potential on the extent of opioid inhibition. (A) The current–voltage relationships of the \(\varepsilon_2/\zeta_1\) channel before and during perfusion of 300 \(\mu\)M meperidine. The measured current responses were normalized to the control current responses at \(-70\) mV before meperidine perfusion (n = 7 or 8). (B) The current–voltage relationships of the \(\varepsilon_2/\zeta_1\) channel before and during perfusion of 300 \(\mu\)M morphine (n = 7 or 8). (C) The effects of the membrane potential on the extent of inhibition of the \(\varepsilon_2/\zeta_1\) channel by 300 \(\mu\)M meperidine, 300 \(\mu\)M morphine, and 1 mM naloxone. The K\(_{\text{app}}\) values (mM) for meperidine, morphine, and naloxone were 2.6 \(\pm\) 0.5, 3.8 \(\pm\) 0.5, and 10.7 \(\pm\) 1.6, respectively, and the \(z_d\) values for those were 0.9 \(\pm\) 0.05, 1.0 \(\pm\) 0.04, and 1.0 \(\pm\) 0.06, respectively (n = 7 or 8).
the e2-N589Q/ζ1 channel was not significantly different from that of the e2/ζ1 channel (Scheffé’s multiple comparison tests, P > 0.99). The IC50 values (μM) of the e2/ζ1, e2-N589Q/ζ1, e2/ζ1-N598Q, and e2-N589Q/ζ1-N598Q channels for meperidine were 206 ± 7 (n = 7), 212 ± 16 (n = 6), 1926 ± 83 (n = 6), and 2194 ± 86 (n = 6), respectively. Similarly, the mutation ζ1-N598Q reduced the sensitivity of the e2/ζ1 channel to morphine and naloxone, whereas the effects of the mutation e2-N589Q were only slight (figs. 6C and 6D). The involvement of the asparagine residue of the ζ1 subunit in determining the opioid sensitivity was further confirmed by the resistance of the e1/ζ1-N598Q channel to opioids (data not shown).

The effects of the mutation ζ1-N598Q on the degree of voltage dependence of block by morphine were examined. The inhibitory effects of morphine (300 μM) on the e2/ζ1-N598Q channel were slight but exhibited voltage dependence (ANOVA, P < 0.0001) (fig. 7A). The degree of voltage dependence of block by morphine (300 μM) was compared between the e2/ζ1 and e2/ζ1-N598Q channels using the Woodhull model (fig. 7B).21 Not only were the Kd(0) values (the affinity of binding) for morphine different between the e2/ζ1 and e2/ζ1-N598Q channels (3.8 ± 0.5 [n = 8] and 6.5 ± 1.0 [n = 6] mM, respectively; t-tests, P < 0.03), but the z0 values (the degree of voltage dependence of block) of the e2/ζ1 and e2/ζ1-N598Q channels were also significantly different (1.0 ± 0.04 [n = 8] and 0.6 ± 0.06 [n = 6], respectively; t tests, P < 0.0001).

Discussion

In the present investigation, we have shown that high concentrations of the naturally occurring opioids morphine and codeine, the phenylpiperidine derivatives meperidine and fentanyl, and the opioid antagonist naloxone inhibit NMDA receptor channels. These results are consistent with previous studies that found that high concentrations of opioids and naloxone protect neurons against central nervous system ischemia and injury and NMDA-induced neurotoxicity.1–3 Recent electrophysiologic and receptor binding studies showed that some opioid agonists, such as meperidine, methadone, and ketobemidone, reduce NMDA-induced depolarization in rat-brain slice preparations at concentrations of 1 mM, and inhibit [3H]MK-801 binding in rat cortical and forebrain membranes.1–6 Furthermore, there are some opioid-related compounds that are already known to be noncompetitive NMDA receptor antagonists. The benzomorphans SKF-10,047 and cyclazocine were shown to exhibit NMDA receptor antagonist properties.24,25 The morphinan opioid levorphanol, its dextrorotatory non-opioid enantiomer dextrorphan, and its O-methyl derivative, dextromethorphan, were also able to selectively antagonize the NMDA-induced neuroexcitation.26,27 These findings suggest that the NMDA receptor antagonist property is a common characteristic of various opioids and related compounds.
The NMDA receptor antagonist activity of opioids should be noted because NMDA receptor channels are suggested to be involved in the changing of opioid efficacy in certain clinical situations. In the pain hypersensitivity states in which opioids are not effective, the coadministration of an NMDA receptor antagonist with opioids was shown to restore the antinociceptive effects of opioids. Furthermore, NMDA receptor antagonists were shown to attenuate or block the development of opioid tolerance and dependence in case of repeated treatment. Therefore, opioids with NMDA receptor antagonist activities may extend the usefulness of opioids in the clinical management of pain. However, the opioids tested in the present investigation could only

The effects of substitution mutations on the opioid sensitivity. (A) The current responses of the e2/ξ1 and e2/ξ1-N598Q channels before (left), during (middle), and after (right) perfusion of 1 mM meperidine. (B) The dose-inhibition relationships for meperidine of the e2/ξ1, e2-N589Q/ξ1, e2/ξ1-N598Q, and e2-N589Q/ξ1-N598Q channels for meperidine were 206 ± 7, 212 ± 16, 1926 ± 85, and 2194 ± 86, respectively, and the Hill coefficient values of those were 0.99 ± 0.03, 1.00 ± 0.04, 1.29 ± 0.09, and 1.31 ± 0.04, respectively (n = 6 or 7). (C) The dose-inhibition relationships for morphine of the e2/ξ1, e2-N589Q/ξ1, e2/ξ1-N598Q, and e2-N589Q/ξ1-N598Q channels. The IC50 values (μM) of the e2/ξ1, e2-N589Q/ξ1, e2/ξ1-N598Q, and e2-N589Q/ξ1-N598Q channels for morphine were 187 ± 9, 285 ± 24, 4626 ± 1920, and 580 ± 67, respectively, and the Hill coefficient values of those were 0.90 ± 0.05, 0.96 ± 0.03, 0.98 ± 0.11, and 0.98 ± 0.03, respectively (n = 7). (D) The dose-inhibition relationships for naloxone of the e2/ξ1, e2-N589Q/ξ1, e2/ξ1-N598Q, and e2-N589Q/ξ1-N598Q channels. The IC50 values (μM) of the e2/ξ1, e2-N589Q/ξ1, e2/ξ1-N598Q, and e2-N589Q/ξ1-N598Q channels for naloxone were 503 ± 34, 426 ± 31, 3474 ± 175, and 1675 ± 152, respectively, and the Hill coefficient values of those were 0.95 ± 0.02, 1.11 ± 0.02, 1.14 ± 0.06, and 1.01 ± 0.02, respectively (n = 7 or 8). The control current responses (nA) of the e2/ξ1, e2-N589Q/ξ1, e2/ξ1-N598Q, and e2-N589Q/ξ1-N598Q channels obtained before perfusion of opioids were 160–890, 180–540, 140–460, and 180–690, respectively.
block NMDA receptor channels at high micromolar concentrations. Plasma concentrations obtained after systemic administration of meperidine and morphine are at most 1–3 mM, and those of fentanyl during high-dose fentanyl anesthesia for cardiac surgery are around 0.1 µM. On the other hand, very high concentrations are obtained in the cerebrospinal fluid (CSF) after epidural or intrathecal administration of opioids in humans. Meperidine concentrations in the CSF after intrathecal injection reach 300–1000 µM, and those after epidural administration come to approximately 100–300 µM because of the rapid absorption across the dural membrane into the CSF. The initial CSF concentrations of morphine following intrathecal administration are in the high micromolar range, and those after epidural administration are about 10 µM. The CSF concentrations of fentanyl after epidural administration are at most 0.1 µM. Thus, the NMDA receptor antagonist property may be clinically significant in the spinal cord following epidural or intrathecal administration of some opioids. Among known opioids with NMDA receptor antagonist properties, methadone and its d- and l-isomers were reported to exhibit relatively high affinities for NMDA receptor channels, approximately similar to those of dextromethorphan. In the rat formalin test, intrathecal administration of the nonopioid d-methadone was shown to have antinociceptive effects as a result of its NMDA receptor antagonist activity.

We have previously shown that the conserved asparagine residue in channel-lining segment M2 of the e2 and ζ1 subunits constitutes the Mg²⁺ block site of NMDA receptor channels, and that PCP, ketamine, SKF-10,047 and (+)MK-801 also act on the Mg²⁺ block site. The effects of mutations on the sensitivity to ketamine were stronger for the ζ1 subunit than for the e2 subunit, whereas mutations in both subunits are required for (+)MK-801 resistance. In the present investigation, the mutation ζ1-N598Q reduced the sensitivity and voltage dependence of opioid inhibition of the e2/ζ1 channel, whereas the mutation e2-N598Q barely affected the sensitivity to opioids. These results support the proposition that the block site of opioids may at least partially overlap with those of the established channel blockers. Furthermore, opioids appear to resemble ketamine rather than (+)MK-801 in terms of the contribution of the conserved asparagine residue of the ζ1 subunit to the block site.

The findings that various opioids and related compounds block NMDA receptor channels raise the question as to which chemical structures of these compounds are responsible for the NMDA receptor channel blocking. Studies on the structural requirements for binding at the PCP site of NMDA receptor channels by analyses of PCP derivatives and (+)MK-801-like molecules have proposed two main requirements of molecules, which correspond to a hydrophobic aromatic moiety and a basic nitrogen atom. Various opioid compounds including both naturally occurring opioids and synthetic compounds such as morphinans, benzomorphans, and phenylpiperidine derivatives, which at first glance seem to be structurally diverse, have a com-

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**Fig. 7.** The effects of the mutation ζ1-N598Q on the voltage dependence of block by morphine. (A) The current–voltage relationships of the e2/ζ1-N598Q channel before and during perfusion of 300 µM morphine. The measured current responses were normalized to the control current responses at −70 mV before morphine perfusion (n = 6). (B) The effects of the membrane potential on the extent of inhibition of the e2/ζ1 and e2/ζ1-N598Q channels by 300 µM morphine. The Kd(0) values (mM) for morphine of the e2/ζ1 and e2/ζ1-N598Q channels were 3.8 ± 0.5 and 6.5 ± 1.0, respectively, and the z0 values of those were 1.0 ± 0.04 and 0.6 ± 0.06, respectively (n = 6–8).
mon vital moiety: an aromatic ring and a nitrogen atom that usually originates from a piperidine ring. Thus, the aromatic ring and the protonated amine of opioid compounds may interact with structural determinants for the PCP binding site of NMDA receptor channels through the hydrophobic interaction and the hydrogen bond, respectively. This proposition is supported by recent structural analyses of the PCP site, which demonstrated that the morphinan derivative dextromethorphan is able to occupy the binding site in a fashion similar to PCP, with its aromatic ring roughly occupying the same region as the phenyl moiety of PCP.

In the present investigation, the effects of opioids on NMDA receptor channels were fully established by the second application of agonists during continuous perfusion of opioids. After opioids were washed out, application of agonists two or three times fully recovered the current responses. This observation is in contrast to that of the potent channel blocker (+)MK-801, which exhibits its progressive and almost irreversible block by sequential application of agonists. The NMDA receptor channel blocking and unblocking kinetics of various noncompetitive antagonists were reported to be highly correlated to their affinities: Lower potency antagonists exhibited faster onset and offset kinetics. Thus, the fast onset and recovery of the block by opioids may be caused by their low affinities for NMDA receptor channels.

Electrophysiologic studies showed that 1 mM meperidine reduced NMDA responses in the rat neonatal spinal cord, whereas meperidine was devoid of antagonist activity in the cerebral cortex. Because the distribution of the four epsilon subunits is distinct in the mature and developing brain, reported differences in meperidine sensitivities in different central nervous system regions seem to be related to differences in the epsilon subunits. In the present investigation, however, meperidine inhibited the epsilon1/epsilon1, epsilon2/epsilon1, epsilon3/epsilon1, and epsilon4/epsilon1 channels to a similar extent. Thus, differences in meperidine sensitivities of NMDA receptor channels in different central nervous system regions cannot be explained only by differences in epsilon subunit species. If the subunit composition of NMDA receptor channels were responsible, differences in a stoichiometry of the epsilon1 subunit might be involved.

In conclusion, high concentrations of various opioid compounds inhibited the current responses of heteromeric NMDA receptor channels in a voltage-dependent manner. The conserved asparagine residue in segment M2 of the epsilon1 subunit was identified as one of molecular determinants of the opioid binding site at NMDA receptor channels. These results suggest that the low-affinity NMDA receptor antagonist activity is not a property specific for a part of opioids as previously considered, but a common characteristic of various opioid compounds. Furthermore, the inhibition was confirmed to be a result of channel block mechanisms at the site, which partially overlaps with those of Mg$^{2+}$ and ketamine. Our results point out the clinical significance of the NMDA receptor antagonist property of some opioids in the spinal cord after local administration and may yield insights into the design of new opioid compounds with higher affinities for NMDA receptor channels.

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