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

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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 $\epsilon 1/\zeta 1$, $\epsilon 2/\zeta 1$, $\epsilon 3/\zeta 1$, and $\epsilon 4/\zeta 1$ 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 (IC_{50}s) 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 Mg^{2+} to the right. Furthermore, replacement of the conserved asparagine residue with glutamine in the channel-lining segment M2 of the ζ1 subunit, which constitutes the block sites of Mg^{2+} and ketamine, reduced the sensitivity to opioids, whereas that of the ε2 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 Mg^{2+} 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.)**

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HIGH concentrations of various opioid agonists and antagonists protect neurons against central nervous system ischemia and injury and neurotoxicity of exogenously applied *N*-methyl-D-aspartate (NMDA).[1–3] More recent electrophysiologic and receptor binding studies have found that some opioid agonists, such as meperidine, methadone, and ketobemidone, reduce NMDA-induced depolarization in rat-brain slice preparations and inhibit [^{3}H]MK-801 (dizocilpine) binding in rat cortical and forebrain membranes.[4–6] These studies suggest that some opioids have NMDA receptor antagonist properties. However, it is uncertain whether the NMDA receptor antagonist activity is a property common to various opioid compounds. In addition, mechanisms of NMDA receptor channel inhibition by opioids remain to be characterized.

Cloning and expression studies have revealed the molecular heterogeneity of the NMDA receptor channel.[7] The mouse NMDA receptor channel is composed of at least two families of subunits, the ε (rat NR2) and ζ (rat NR1) subfamilies of the glutamate receptor channel, which share amino acid sequence homology with the subunits of the α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA)– or kainate-selective glutamate receptor channels. The functional properties of the ε/ζ heteromeric NMDA receptor channels are determined by the constituting ε subunit species (ε1–ε4). The heteromeric $\epsilon 1/\zeta 1$, $\epsilon 2/\zeta 1$, $\epsilon 3/\zeta 1$, and $\epsilon 4/\zeta 1$ channels exhibit different affinities for agonists and different sensitivities to Mg^{2+} block and competitive and noncompetitive antagonists. The ε1 and ζ1 subunit mRNAs are widely distributed in the brain, whereas the ε2 subunit mRNA is expressed abundantly in the forebrain. The ε3 subunit mRNA is predominantly found in the cerebellum, but the ε4 subunit mRNA is weakly expressed in the diencephalon and the brainstem. Reports on expression patterns in the adult spinal cord are controversial. The ζ1, ε1, and ε2 subunit mRNAs are the predominant transcripts detected in the mouse cervical cord,[8] whereas the NR1-ζ1, NR2C-ε3, and NR2D-ε4 subunit mRNAs are found in the rat lumbar spinal cord.[9]

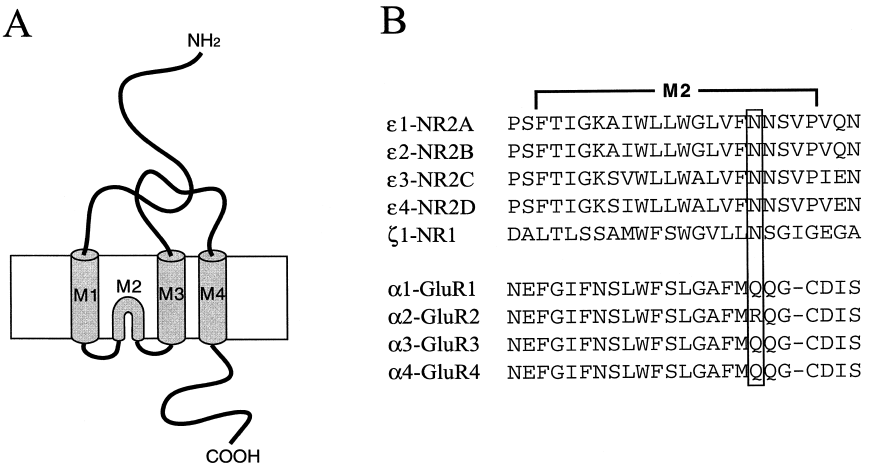

Fig. 1. Schematic representations of the proposed structure of glutamate receptor channel subunits. (*A*) Three transmembrane segment topology model. (*B*) Amino acid sequences of segment M2 of the glutamate receptor channel subunits selective for NMDA (ϵ1, ϵ2, ϵ3, ϵ4, and ζ1) and those selective for 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 divalent cations.

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).[7] Site-directed mutagenesis has revealed that the conserved asparagine (N) residues in segment M2 of the ϵ and ζ subunits govern both Mg^{2+} block and Ca^{2+} permeability of NMDA receptor channels, thus indicating that segment M2 constitutes the ion-channel pore of NMDA receptor channels.[10,11] The position of this asparagine residue corresponds to that of glutamine (Q) or arginine (R) of the α subunits, which determines the Ca^{2+} permeability of the AMPA-selective glutamate receptor channel [12] (fig. 1B).

In the present investigation, we examined the effects and mechanisms of action of various opioid compounds (phenylpiperidine derivatives meperidine and fentanyl, naturally occurring opioids morphine and codeine, and opioid antagonist naloxone) on the ϵ1/ζ1, ϵ2/ζ1, ϵ3/ζ1 and ϵ4/ζ1 heteromeric NMDA channels expressed in *Xenopus* oocytes. Furthermore, the action sites of opioids on NMDA receptor channels were investigated by site-directed mutagenesis.

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 ^{7}mGpppG. 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,[13] pSPGRϵ2,[14] pSPGRϵ3,[15] pSPGRϵ4,[16] and pSPGRζ1,[15] respectively. The ϵ2, ϵ2-N589Q, ζ1, and ζ1-N598Q subunit-specific mRNAs (for the expression of the ϵ2-N589Q/ζ1, ϵ2/ζ1-N598Q, and ϵ2-N589Q/ζ1-N598Q channels) were synthesized using pBKSAϵ2,[17] pBKSAϵ2-N589Q,[11] pBKSAζ1,[18] and pBKSAζ1-N598Q,[11] respectively. The α1 and α2 subunit-specific mRNAs for the α1/α2 AMPA-selective glutamate receptor channel were synthesized using pSPGR1 and pSPGR2, respectively.[19]

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-N598Q, and ϵ2-N589Q/ζ1-N598Q 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-micropipette voltage clamp.[19] The current responses of the wild-type and mutant ϵ/ζ channels to 10 μM L-glutamate plus 10 μM glycine (almost saturating concentrations for all ϵ/ζ channels) were measured in Ba^{2+}-Ringer's solution to minimize the effects of secondarily activated Ca^{2+}-dependent Cl^- currents.[20] 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. Preapplication 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 $BaCl_2$, and 10 mM HEPES-NaOH (*p*H 7.2). Normal frog Ringer's solution contained 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, and 10 mM HEPES-NaOH (*p*H 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 $\epsilon 2/\zeta 1$ channel by 5 ± 1% (mean ± SEM, n = 4).

Statistical Analysis

The inhibitor concentration for half-control response (IC_{50}) and the Hill coefficient values for opioids of the ϵ/ζ channel were calculated according to the equation $Ropi = 1/[1 + (O/IC_{50})^n]$, where *Ropi* represents the relative response, *O* the concentration of opioids, and *n* the Hill coefficient. The agonist concentration for half-control response (EC_{50}) of the ϵ/ζ channel was calculated according to the equation $Rago = Fopi/[1 + (EC_{50}/A)^n]$, 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[21] by fitting the data to the equation $Ropi = 1/[1 + (O/K_{d(O)}\exp(z\delta FE/RT))]$, where *Ropi* represents the relative response, *O* the concentration of opioids, $K_{d(O)}$ the equilibrium dissociation constant of opioids at a membrane potential of 0 mV, *z* the charge of opioids, δ 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 ϵ/ζ Heteromeric NMDA Receptor Channels

Four kinds of heteromeric NMDA receptor channels, the $\epsilon 1/\zeta 1$, $\epsilon 2/\zeta 1$, $\epsilon 3/\zeta 1$, and $\epsilon 4/\zeta 1$ channels, were expressed in *Xenopus* oocytes by the injection of respective subunit-specific mRNAs synthesized *in vitro* from cloned cDNAs. The sensitivities of these ϵ/ζ 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 ϵ/ζ 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 $\epsilon 1/\zeta 1$, $\epsilon 2/\zeta 1$, $\epsilon 3/\zeta 1$, and $\epsilon 4/\zeta 1$ channels to a similar extent in a concentration-dependent manner. The IC_{50} values (μM) of the $\epsilon 1/\zeta 1$, $\epsilon 2/\zeta 1$, $\epsilon 3/\zeta 1$, and $\epsilon 4/\zeta 1$ channels for meperidine were 233 ± 14 (n = 6), 206 ± 7 (n = 7), 264 ± 9 (n = 6), and 273 ± 12 (n = 6), respectively. The $\epsilon 2/\zeta 1$ channel was more sensitive to meperidine than the $\epsilon 3/\zeta 1$ and $\epsilon 4/\zeta 1$ channels ($\log[IC_{50}]$ 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 $\alpha 1/\alpha 2$ 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 ϵ/ζ channels in

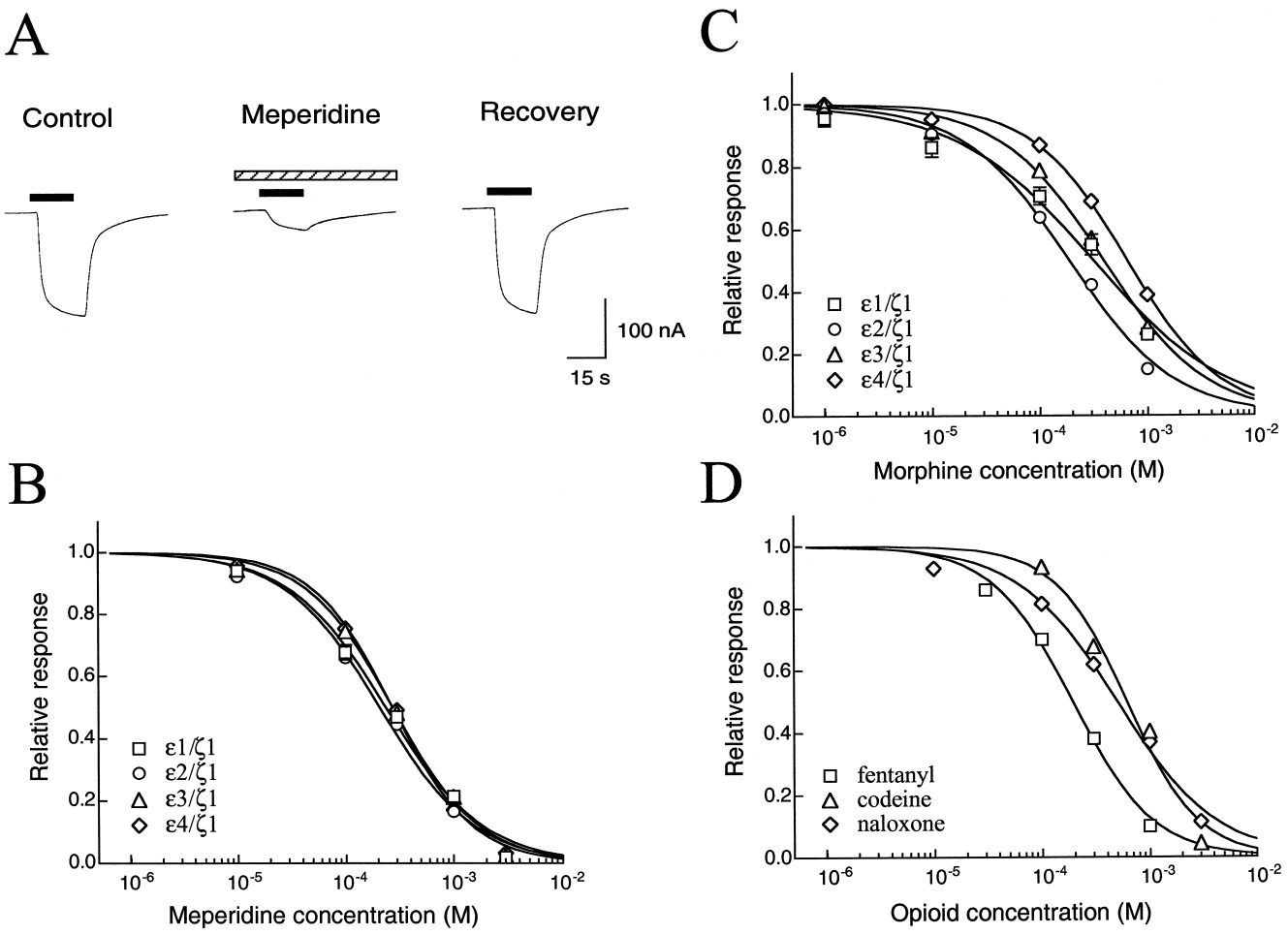

Fig. 2. The effects of various opioids on heteromeric NMDA receptor channels. (*A*) The current responses of the ε1/ζ1 channel before (*left*), during (*middle*), and after (*right*) perfusion of 1 mM meperidine. Inward current is downward. The duration of bath application of 10 μM L-glutamate plus 10 μM glycine is indicated by bars without taking into account the dead-space time in the perfusion system (approximately 2 s). The duration of meperidine perfusion is indicated by the *batched column*. (*B*) The dose-inhibition relationships for meperidine of four heteromeric ε/ζ channels. Each point represents the mean ± SEM of measurements on six or seven oocytes; SEMs are indicated by bars if larger than the symbols. The IC_{50} values (μM, mean ± SEM) of the ε1/ζ1, ε2/ζ1, ε3/ζ1, and ε4/ζ1 channels for meperidine were 233 ± 14, 206 ± 7, 264 ± 9, and 273 ± 12, respectively, and the Hill coefficient values of those were 1.00 ± 0.03, 0.99 ± 0.03, 1.14 ± 0.01, and 1.21 ± 0.02, respectively. (*C*) The dose-inhibition relationships for morphine of four heteromeric ε/ζ channels. The IC_{50} values (μM) of the ε1/ζ1, ε2/ζ1, ε3/ζ1, and ε4/ζ1 channels for morphine were 321 ± 48, 187 ± 9, 392 ± 27, and 650 ± 24, respectively, and the Hill coefficient values of those were 0.72 ± 0.07, 0.90 ± 0.05, 0.92 ± 0.06 and 1.01 ± 0.02, respectively (n = 6 or 7). (*D*) The effects of fentanyl, codeine and naloxone on the ε2/ζ1 channel. The IC_{50} values (μM) of the ε2/ζ1 channel for fentanyl, codeine, and naloxone were 192 ± 9, 613 ± 25, and 503 ± 34, respectively, and the Hill coefficient values for those were 1.18 ± 0.03, 1.32 ± 0.03, and 0.95 ± 0.02, respectively (n = 7 or 8). The control current responses (nA) of the ε1/ζ1, ε2/ζ1, ε3/ζ1, and ε4/ζ1 channels obtained before perfusion of opioids were 160–788, 165–888, 65–440, and 80–370, respectively.

a dose-dependent manner. The sensitivities to morphine varied between the four ε/ζ channels ($\log[IC_{50}]$ values were compared by ANOVA; $P < 0.0001$; fig. 2C). The IC_{50} values (μM) of the ε1/ζ1, ε2/ζ1, ε3/ζ1 and ε4/ζ1 channels were 321 ± 48 (n = 7), 187 ± 9 (n = 7), 392 ± 27 (n = 6) and 650 ± 24 (n = 7), respectively. The ε2/ζ1 channel was the most sensitive to morphine among the four ε/ζ 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 (μM) of the ε2/ζ1 channel for fentanyl, codeine, and naloxone were 192 ± 9 (n = 8), 613 ± 25 (n = 7), and 503 ± 34 (n = 8), 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 ϵ/ζ channel for agonists. The dose–response relationships of the $\epsilon 2/\zeta 1$ 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 EC_{50} values (μM) of the $\epsilon 2/\zeta 1$ 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) ($\log[EC_{50}]$ 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 $\epsilon 2/\zeta 1$ channel without affecting the EC_{50} values (fig. 3B). These results suggest the noncompetitive 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 $\epsilon 2/\zeta 1$ channel before and during perfusion of 300 μM meperidine. Meperidine inhibition of the $\epsilon 2/\zeta 1$ 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 $\epsilon 2/\zeta 1$ 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 $\epsilon 2/\zeta 1$ channel in a voltage-dependent manner (fig. 4B). The degree of voltage dependence of inhibition of the $\epsilon 2/\zeta 1$ channel by meperidine (300 μM), morphine (300 μM), and naloxone (1 μM) was compared using the Woodhull model [21] (fig. 4C). Although the $K_{d(O)}$ 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 $z\delta$ values (the degree of voltage dependence of block) for those were not significantly different

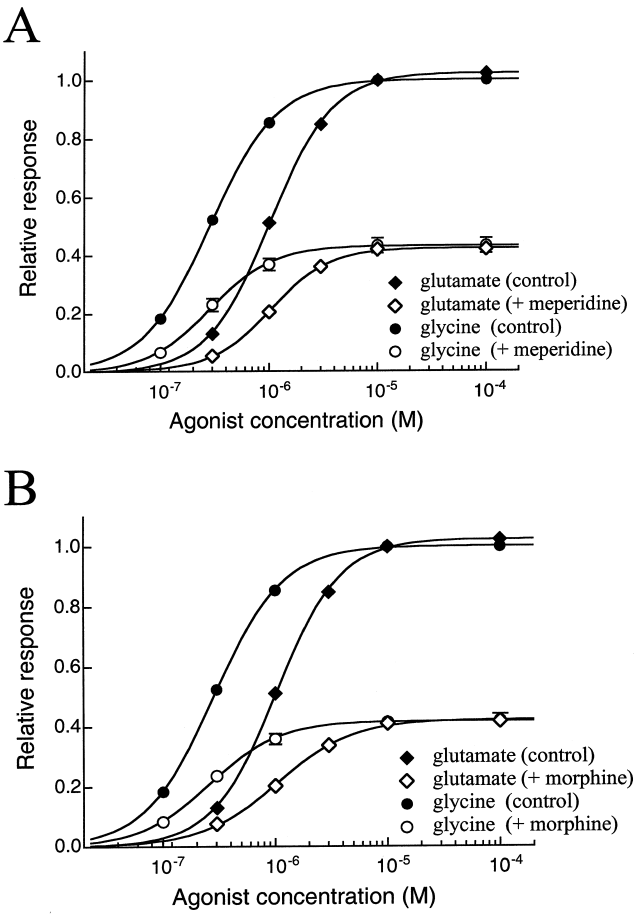

Fig. 3. The effects of opioids on the dose-response relationships of the $\epsilon 2/\zeta 1$ channel for L-glutamate and glycine. (*A*) Dose–response relationships of the $\epsilon 2/\zeta 1$ channel for L-glutamate in the presence of 10 μM glycine and those for glycine in the presence of 10 μM L-glutamate before and during perfusion of 300 μM meperidine. The measured current responses were normalized to the control current responses to 10 μM L-glutamate plus 10 μM glycine. The EC_{50} values (μM) of the $\epsilon 2/\zeta 1$ channel for L-glutamate before and during perfusion of 300 μM meperidine were 1.03 ± 0.02 and 1.04 ± 0.04, respectively, and the Hill coefficient values for those were 1.53 ± 0.04 and 1.62 ± 0.10, respectively (n = 6 or 7). The EC_{50} values (μM) of the $\epsilon 2/\zeta 1$ channel for glycine before and during perfusion of 300 μM meperidine were 0.29 ± 0.01 and 0.29 ± 0.01, respectively, and the Hill coefficient values for those were 1.42 ± 0.05 and 1.56 ± 0.07, respectively (n = 6 or 7). (*B*) Dose–response relationships of the $\epsilon 2/\zeta 1$ channel for L-glutamate in the presence of 10 μM glycine and those for glycine in the presence of 10 μM L-glutamate before and during perfusion of 300 μM morphine. The EC_{50} values (μM) of the $\epsilon 2/\zeta 1$ channel for L-glutamate and glycine during perfusion of 300 μM morphine were 1.05 ± 0.03 and 0.26 ± 0.02, respectively, and the Hill coefficient values for those were 1.38 ± 0.05 and 1.44 ± 0.09, respectively (n = 6 or 7).

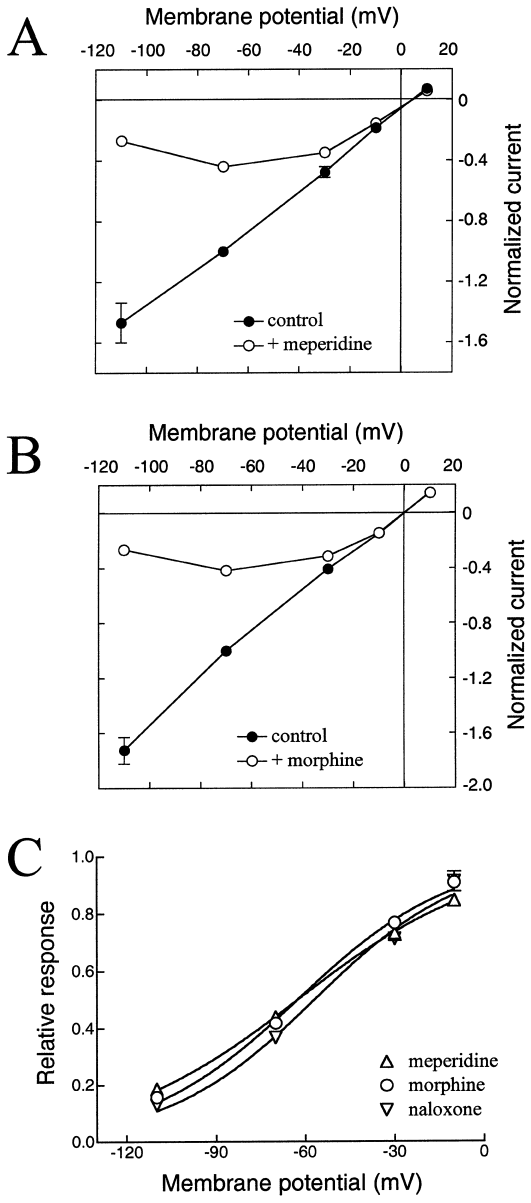

Fig. 4. The effects of the membrane potential on the extent of opioid inhibition. (*A*) The current–voltage relationships of the $\epsilon 2/\zeta 1$ channel before and during perfusion of 300 μM meperidine. The measured current responses were normalized to the control current responses at −70 mV before meperidine perfusion (n = 7 or 8). (*B*) The current–voltage relationships of the $\epsilon 2/\zeta 1$ channel before and during perfusion of 300 μM morphine (n = 7 or 8). (*C*) The effects of the membrane potential on the extent of inhibition of the $\epsilon 2/\zeta 1$ channel by 300 μM meperidine, 300 μM morphine, and 1 mM naloxone. The $K_{d(0)}$ values (mM) for meperidine, morphine, and naloxone were 2.6 ± 0.5, 3.8 ± 0.5, and 10.7 ± 1.6, respectively, and the $z\delta$ values for those were 0.9 ± 0.05, 1.0 ± 0.04, and 1.0 ± 0.06, respectively (n = 7 or 8).

(0.9 ± 0.05 [n = 7], 1.0 ± 0.04 [n = 8], and 1.0 ± 0.06 [n = 8], respectively; ANOVA, $P > 0.07$).

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 μM) and morphine (300 μM) on the sensitivity of the $\epsilon 2/\zeta 1$ channel to the Mg^{2+} block (fig. 5A). Mg^{2+} inhibited the current responses of the $\epsilon 2/\zeta 1$ channel in a dose-dependent manner with IC_{50} values of 15.5 ± 1.0 μM (n = 8). Meperidine and morphine shifted the inhibition curve of Mg^{2+} to the right (fig. 5B). The IC_{50} values (μM) for Mg^{2+} during perfusion of meperidine and morphine were 30.0 ± 1.9 (n = 7) and 37.0 ± 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 Scheffé'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 $\epsilon 2$ and $\zeta 1$ subunits (the asparagine 589 of the $\epsilon 2$ 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 $\epsilon 2$ and $\zeta 1$ subunits (the mutations $\epsilon 2$-N589Q and $\zeta 1$-N598Q, respectively) on the sensitivity to opioids. The mutation $\zeta 1$-N598Q reduced the sensitivity to meperidine (fig. 6A). The $\epsilon 2/\zeta 1$-N598Q and $\epsilon 2$-N589Q/$\zeta 1$-N598Q channels were more resistant to meperidine than the $\epsilon 2/\zeta 1$ and $\epsilon 2$-N589Q/$\zeta 1$ channels ($\log[IC_{50}]$ values were compared by ANOVA followed by Scheffé's multiple comparison tests, $P <$ 0.0001) (fig. 6B). On the other hand, the sensitivity of

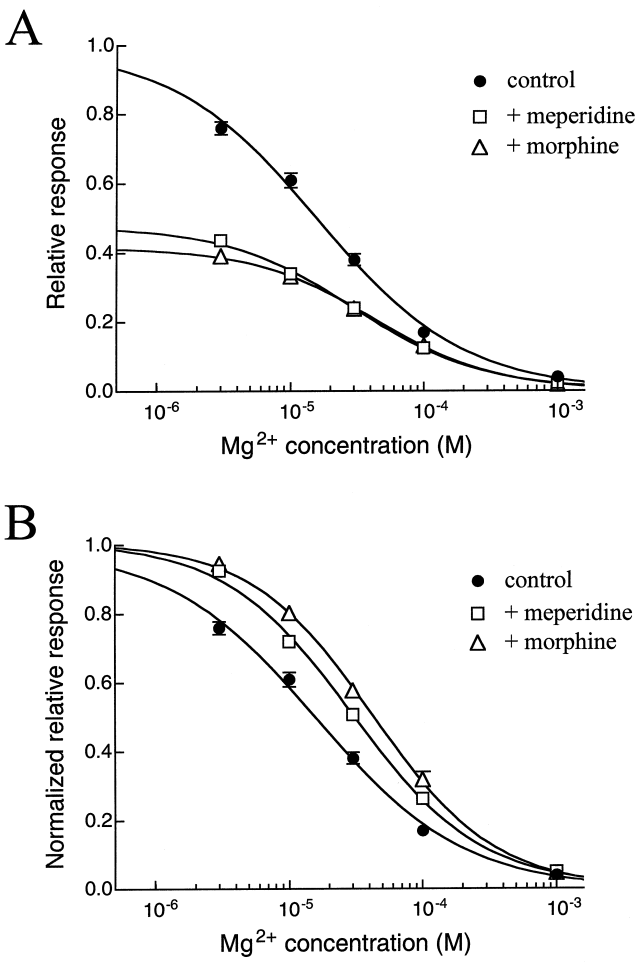

Fig. 5. The effects of meperidine and morphine on the sensitivity to the Mg^{2+} block. (*A*) The dose-inhibition relationships of the $\epsilon 2/\zeta 1$ channel for Mg^{2+} before and during perfusion of meperidine (300 μM) and morphine (300 μM). (*B*) The normalized dose-inhibition relationships of the $\epsilon 2/\zeta 1$ channel for Mg^{2+}. The measured current responses during perfusion of opioids and Mg^{2+} were normalized to the current responses in the absence of Mg2+ during opioid perfusion. The IC_{50} values (μM) of the $\epsilon 2/\zeta 1$ channel for Mg^{2+} before, and during perfusion of meperidine and morphine were 15.5 ± 1.0, 30.0 ± 1.9, and 37.0 ± 2.5, respectively, and the Hill coefficient values for those were 0.81 ± 0.06, 0.89 ± 0.03, and 1.05 ± 0.04, respectively (n = 7 or 8).

the ϵ2-N589Q/ζ1 channel was not significantly different from that of the $\epsilon 2/\zeta 1$ channel (Scheffé's multiple comparison tests, $P > 0.99$). The IC_{50} values (μM) of the $\epsilon 2/\zeta 1$, ϵ2-N589Q/ζ1, $\epsilon 2/\zeta$1-N598Q, and ϵ2-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 $\epsilon 2/\zeta 1$ channel to morphine and naloxone, whereas the effects of the mutation ϵ2-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 $\epsilon 1/\zeta$1-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 $\epsilon 2/\zeta$1-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 $\epsilon 2/\zeta 1$ and $\epsilon 2/\zeta$1-N598Q channels using the Woodhull model (fig. 7B).[21] Not only were the $K_{d(0)}$ values (the affinity of binding) for morphine different between the $\epsilon 2/\zeta 1$ and $\epsilon 2/\zeta$1-N598Q channels (3.8 ± 0.5 [n = 8] and 6.5 ± 1.0 [n = 6] mM, respectively; *t*-tests, $P < 0.03$), but the $z\delta$ values (the degree of voltage dependence of block) of the $\epsilon 2/\zeta 1$ and $\epsilon 2/\zeta$1-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 [^{3}H]MK-801 binding in rat cortical and forebrain membranes.[4–6] 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 nonopioid 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.

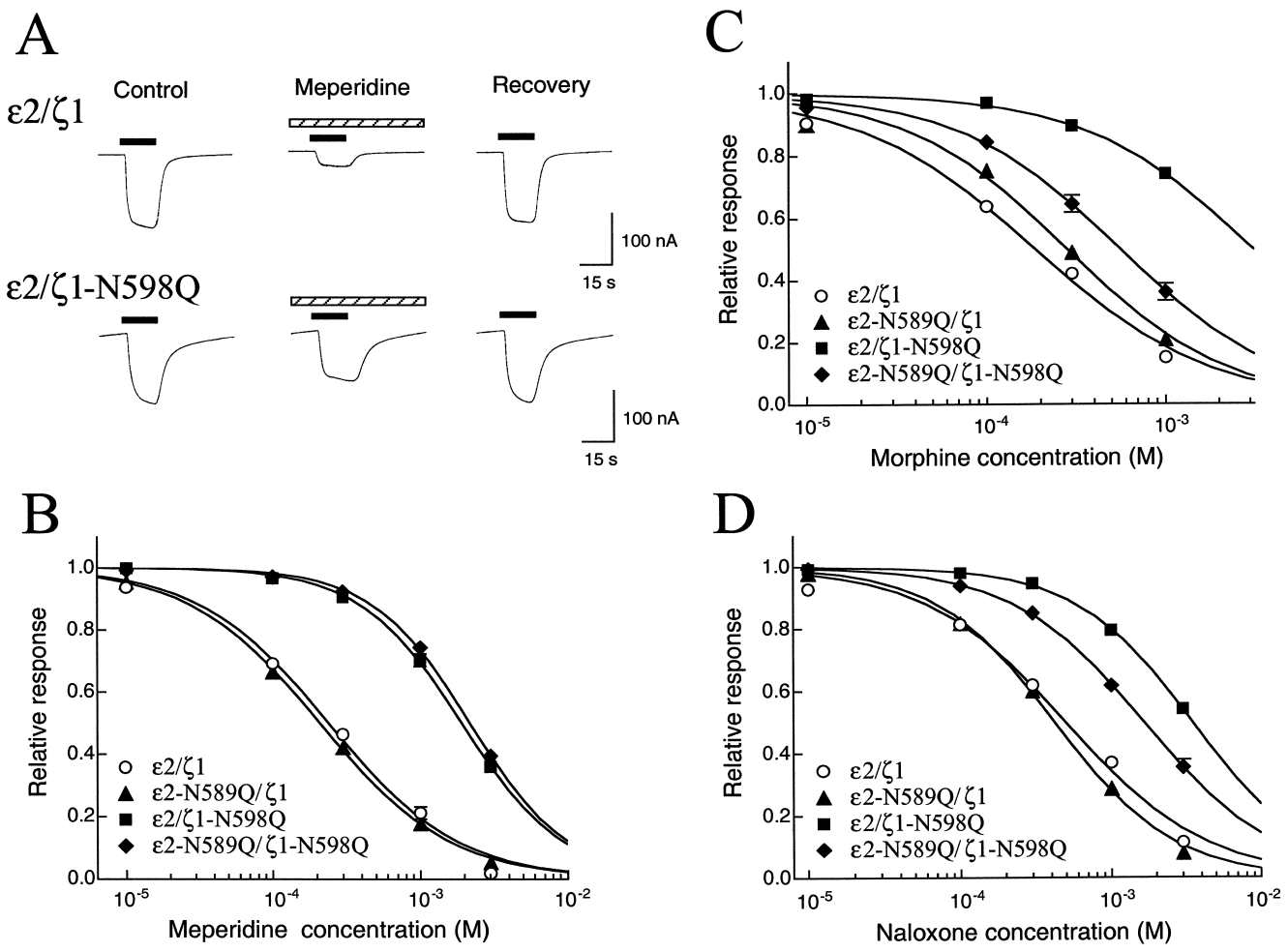

Fig. 6. The effects of substitution mutations on the opioid sensitivity. (*A*) The current responses of the ε2/ζ1 and ε2/ζ1-N598Q channels before (*left*), during (*middle*), and after (*right*) perfusion of 1 mM meperidine. (*B*) The dose-inhibition relationships for meperidine of the ε2/ζ1, ε2-N589Q/ζ1, ε2/ζ1-N598Q, and ε2-N589Q/ζ1-N598Q channels. The IC_{50} values (μM) of the ε2/ζ1, ε2-N589Q/ζ1, ε2/ζ1-N598Q, and ε2-N589Q/ζ1-N598Q channels for meperidine were 206 ± 7, 212 ± 16, 1926 ± 83, 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 ε2/ζ1, ε2-N589Q/ζ1, ε2/ζ1-N598Q, and ε2-N589Q/ζ1-N598Q channels. The IC_{50} values (μM) of the ε2/ζ1, ε2-N589Q/ζ1, ε2/ζ1-N598Q, and ε2-N589Q/ζ1-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 ε2/ζ1, ε2-N589Q/ζ1, ε2/ζ1-N598Q, and ε2-N589Q/ζ1-N598Q channels. The IC_{50} values (μM) of the ε2/ζ1, ε2-N589Q/ζ1, ε2/ζ1-N598Q, and ε2-N589Q/ζ1-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 ε2/ζ1, ε2-N589Q/ζ1, ε2/ζ1-N598Q, and ε2-N589Q/ζ1-N598Q channels obtained before perfusion of opioids were 160–890, 180–540, 140–460, and 180–690, respectively.

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.[28,29] Furthermore, NMDA receptor antagonists were shown to attenuate or block the development of opioid tolerance and dependence in case of repeated treatment.[30,31] 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

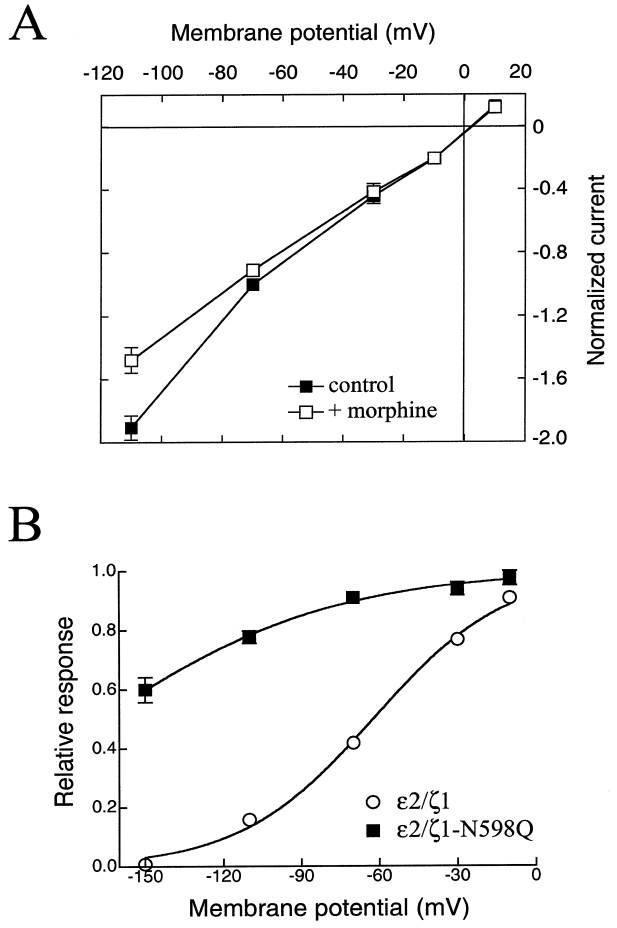

Fig. 7. The effects of the mutation ζ1-N598Q on the voltage dependence of block by morphine. (*A*) The current–voltage relationships of the ε2/ζ1-N598Q channel before and during perfusion of 300 μM morphine. The measured current responses were normalized to the control current responses at −70 mV before morphine perfusion (n = 6). (*B*) The effects of the membrane potential on the extent of inhibition of the ε2/ζ1 and ε2/ζ1-N598Q channels by 300 μM morphine. The $K_{d(0)}$ values (mM) for morphine of the ε2/ζ1 and ε2/ζ1-N598Q channels were 3.8 ± 0.5 and 6.5 ± 1.0, respectively, and the zδ values of those were 1.0 ± 0.04 and 0.6 ± 0.06, respectively (n = 6–8).

block NMDA receptor channels at high micromolar concentrations. Plasma concentrations obtained after systemic administration of meperidine and morphine are at most 1–3 μM,[32,33] and those of fentanyl during high-dose fentanyl anesthesia for cardiac surgery are around 0.1 μM.[34,35] 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.[36,37] The initial CSF concentrations of morphine following intrathecal administration are in the high micromolar range, and those after epidural administration are about 10 μM;[38,39] the CSF concentrations of fentanyl after epidural administration are at most 0.1 μM.[40] 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.[4,6] 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.[41]

We have previously shown that the conserved asparagine residue in channel-lining segment M2 of the ε2 and ζ1 subunits constitutes the Mg^{2+} block site of NMDA receptor channels, and that PCP, ketamine, SKF-10,047 and (+)MK-801 also act on the Mg^{2+} block site.[11,13] The effects of mutations on the sensitivity to ketamine were stronger for the ζ1 subunit than for the ε2 subunit, whereas mutations in both subunits are required for (+)MK-801 resistance.[11,13] In the present investigation, the mutation ζ1-N598Q reduced the sensitivity and voltage dependence of opioid inhibition of the ε2/ζ1 channel, whereas the mutation ε2-N589Q 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.[42,43] 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-

mon vital moiety: an aromatic ring and a nitrogen atom that usually originates from a piperidine ring.[44] 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.[43]

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 the progressive and almost irreversible block by sequential application of agonists.[13] 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.[45] 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.[4] Because the distribution of the four ϵ subunits is distinct in the mature and developing brain,[7] reported differences in meperidine sensitivities in different central nervous system regions seem to be related to differences in the ϵ subunits. In the present investigation, however, meperidine inhibited the $\epsilon 1/\zeta 1$, $\epsilon 2/\zeta 1$, $\epsilon 3/\zeta 1$, and $\epsilon 4/\zeta 1$ 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 ϵ subunit species. If the subunit composition of NMDA receptor channels were responsible, differences in a stoichiometry of the $\zeta 1$ 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 $\zeta 1$ 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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