The Mechanism of Action for the Block of NMDA Receptor Channels by the Opioid Peptide Dynorphin

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Dynorphin is one of the endogenous opioids that modulates the excitability of nociceptive (pain-sensing) neurons. We have shown recently that dynorphin blocks NMDA-activated currents directly without the participation of κ -opioid receptors. In order to understand the mechanism underlying this novel action of dynorphin, we examined, in detail, the interactions between dynorphin and NMDA receptors in isolated trigeminal neurons. Dynorphin reversibly blocks NMDA-activated current (I_{NMDA}). The onset and recovery of the block were determined with concentration jump experiments. The association rate (k_+) of dynorphin(1-17) is $4.9 \times 10^6 \text{ sec}^{-1} \text{ M}^{-1}$ and the dissociation rate (k_-) is 7.5 sec^{-1} . The apparent dissociation constant (K_D) of dynorphin, calculated from these rate constants, is $1.6 \text{ }\mu\text{M}$. Dynorphin does not change the EC_{50} of NMDA, nor the potentiating action of glycine. The binding site for dynorphin is distinct from that of Zn^{2+} or H^+ . Upon treatment with the disulfide reducing agent dithiothreitol (DTT), NMDA receptors become less susceptible to dynorphin block. The affinity of dynorphin for the modified NMDA receptors is reduced by 2.7-fold. In analyses of single NMDA channels in cell-free patches, we found that dynorphin shortens the mean open time, decreases the probability of opening of NMDA channels, but has no effect on the single channel conductance. These results suggest that dynorphin interacts with a site conformationally linked with the redox site(s) on the NMDA receptor, thus altering the gating properties of the channel.

[Key words: dynorphin, trigeminal neurons, pain, NMDA receptor channel, nonopioid, κ -opioid]

Trigeminal and spinal dorsal horn neurons in the spinothalamic tract (STT) relay nociceptive and temperature information to the brain. These neurons receive noxious inputs from primary afferent fibers, integrate modulatory influences from descending and local circuits and send the information to the thalamus and the somatosensory cortex (Zieglgansberger, 1986; Fields, 1987; Willis and Coggeshall, 1991). The major excitatory transmitters mediating the synaptic transmission in this nociceptive pathway are glutamate and aspartate. They are located in afferent fibers,

interneurons (Schneider and Perl, 1985; Battaglia and Rustioni, 1988; Schneider and Perl, 1988), and in 50% of the terminals apposing the somata and dendrites of the projection neurons (Westlund et al., 1992). In response to noxious stimuli, glutamate and aspartate are released (Potashner and Tran, 1984; Skilling et al., 1988) and activate NMDA and non-NMDA receptors (Jahr and Jessell, 1985; Aanonsen and Wilcox, 1987; Gerber and Randić, 1989; Gu and Huang, 1989; Gu and Huang, 1990; Yoshimura and Jessell, 1990; Chen and Huang, 1992a; Dougherty and Willis, 1992; Dougherty et al., 1992).

The endogenous opioid, dynorphin, has been shown to contribute to analgesia under physiological conditions (Millan, 1990). Immunolabeled dynorphin cells are concentrated in laminae I and II of the dorsal horn. Some can be seen in deeper laminae (IV–VI and X) (Cruz and Basbaum, 1985; Miller and Seybold, 1987; Ruda et al., 1988; Weihe et al., 1989). Kappa (κ)-receptor binding is found mostly in lamina II (Gouarderes et al., 1985; Morris and Herz, 1987). There is considerable mismatch between the anatomical distribution of dynorphin and the κ -receptor binding, similar to those of μ - and δ -opioid peptides (Mansour et al., 1988; Mansour and Watson, 1993).

Responding to high frequency stimulation of unmyelinated afferent fibers, dynorphin(1-17), a primary product of prodynorphin (Cone et al., 1983), is released in laminae I and V–VI (Hutchison et al., 1990) and can modulate synaptic transmission in the dorsal horn. Dynorphin and the κ -opioid agonist U50,488H were found to reduce the firing frequency of dorsal horn neurons evoked by glutamate application (Willcockson et al., 1986), by electrical C-fiber stimulation (Knox and Dickenson, 1987; Caudle and Isaac, 1988), or by noxious mechanical and thermal stimuli (Fleetwood-Walker et al., 1988; Millan, 1989; Hope et al., 1990). Behavioral studies have shown that intrathecal administration of dynorphin(1-17) increases the latency of thermally evoked tail flick reflexes (Herman and Goldstein, 1985; Spampinato and Candeletti, 1985) (for review, see Yaksh, 1993). In addition to the inhibitory effects, dynorphin sometimes exerts excitatory actions on these neurons. Application of dynorphin was found to increase the receptive field, to enhance the activity of dorsal horn neurons in response to C-fiber or mechanical stimulation (Knox and Dickenson, 1987; Hylden et al., 1991) and to enhance the glutamate-evoked firing of STT neurons (Willcockson et al., 1986). Not all of these dynorphin actions are mediated by κ -opioid receptors; many of the dynorphin effects can not be blocked by high doses of the opioid receptor antagonist naloxone (Willcockson et al., 1986; Knox and Dickenson, 1987; Caudle and Isaac, 1988; Hylden et al., 1991).

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Dynorphin and NMDA receptors are thought to play a pivotal role in the sensitization of nociceptive neurons (Dubner and Ruda, 1992). Upon receiving repetitive stimuli to small diameter afferent fibers, the dorsal horn neurons generate prolonged depolarization and/or fire with increasing frequency, that is, "windup" (Wall and Woolf, 1984; Dickenson and Sullivan, 1987; Yoshimura and Jessel, 1989). This sensitization of central neurons can not be initiated nor be maintained when the activation of NMDA receptors is blocked by ($\pm$)-2-amino-5-phosphonovaleric acid (APV) (Davies and Lodge, 1987; Dickenson and Sullivan, 1987; Headley et al., 1987; Woolf and Thompson, 1991). Following tissue injury or inflammation, dorsal horn neurons develop hypersensitivity to innocuous or noxious stimuli and the receptive field size for these neurons increases (Hylden et al., 1991). In addition to the change in electrical activity, the expression of opioids is also enhanced after tissue injury. Enkephalin increases about 50%; dynorphin increases up to four-fold (Millan et al., 1986; Iadarola et al., 1988; Weihe et al., 1989; Kajander et al., 1990; Dubner and Ruda, 1992; Millan, 1993). The consequence of this large increase in dynorphin expression is not clear at the moment.

The cellular mechanisms of dynorphin actions have been studied extensively (Huang, 1995). Presynaptically, dynorphin changes the release of glutamate, noradrenaline, SP, and enkephalin (Werling et al., 1987; Xu and Gintzler, 1989; Gannon and Terrian, 1991; Suarez-Roca and Maixner, 1993; Wagner et al., 1993; Weisskopf et al., 1993). Postsynaptically, dynorphin hyperpolarizes the membrane by opening K^+ channels (Grudt and Williams, 1993) or closing Ca^{2+} channels (Gross and MacDonald, 1987; Bean, 1989). We have recently found that dynorphin acts directly on NMDA receptors without the involvement of opioid receptors (Chen and Huang, 1992b, 1993; Chen et al., 1995). To better understand the mechanism of dynorphin action, we further studied the kinetics of dynorphin binding, analyzed the modulation of single NMDA channel activity by dynorphin, and explored the site of action for dynorphin.

Materials and Methods

Acutely dissociated trigeminal neurons were isolated from 9–16 d old Long Evans rats as previously described (Huang, 1989; Gu and Huang, 1991). In brief, the lower medulla and upper cervical regions of the spinal cord were removed and put in an ice-cold, oxygenated dissecting solution, consisting of (mM) NaCl (120), KCl (10), $CaCl_2$ (1), $MgCl_2$ (6), glucose (10), and PIPES (10) (pH = 7.15) (osmolality = 305–315 mOsm). The tissue was cut into 300 μ m thick horizontal slices with a vibratome slicer and incubated in the dissecting solution for 15–30 min at 34.5°C. The slices were then incubated with 0.75 mg/ml protease III (Sigma) in the dissecting solution for 10 min at 34.5°C. After incubation, the tissue was washed with enzyme-free dissecting solution and stored at room temperature. Prior to an experiment, the spinal trigeminal nuclei in the caudal medulla were isolated from a tissue slice with a scalpel. Neurons were dissociated by triturating the tissue with a series of fire-polished Pasteur pipettes.

Whole-cell and single-channel currents were recorded using the patch clamp technique. The external solution contained (mM) NaCl (140), KCl (4), glucose (10), HEPES (10) (pH = 7.4), and $CaCl_2$ (2). Unless otherwise stated, 100 μ M NMDA and 2 μ M glycine were used. The internal solution contained (mM) Cs methanesulfonate (125), CsCl (15), glucose (10), BAPTA (11), $CaCl_2$ (1), and HEPES (10) (pH = 7.2). The free intracellular Ca^{2+} concentration was calculated to be 1.6×10^{-8} M. Mg-ATP (5 mM), leupeptin (400 μ M), and GTP (200 μ M) were added to the internal solution to prevent the rundown. All the chemicals were ultrapure grade. The amino acids and various dynorphins were delivered to the recorded cell using the fast perfusion technique (Gu and Huang, 1991). The solution exchange time was measured from the time taken for the whole-cell kainate currents to reach a new steady state as the extracellular Na^+ was switched from 140 to 70 mM. The solution ex-

Figure 1. Dynorphin inhibits NMDA-activated currents. *A*, Dynorphin(1-32), at 0.25 μ M, reduced I_{NMDA} to 40% of the control. The currents returned to the original amplitude when dynorphin was washed out. *B*, Time course of dynorphin block. The blocking effect of dynorphin was rapid and the block was reversed after dynorphin was removed from the bath media. The same actions of dynorphin could be repeatedly observed in the same cell. The holding potential was at -70 mV.

change was completed within 10 msec. Unless specified, the length of NMDA application was ~ 12 sec. To avoid desensitization or rundown of the whole-cell I_{NMDA} , NMDA was washed out completely after each application, and NMDA pulses were applied once every 2 min. The least squares method was used to fit the theoretical dose-response curves to the experimental data. The average data values are expressed as mean $\pm$ SE.

Whole-cell currents were filtered at 2 kHz and sampled at 200 μ sec. Single-channel currents were recorded from outside-out patches; these signals were filtered at 2 kHz and sampled at 100 μ sec. The amplitude of a single NMDA-activated channel was obtained from amplitude histograms of all the digitized points. Only the main conductance level (37 pS) was included in our analyses. Transition from a closed to an open state was detected when the current level was above 50% of the main conductance level and lasted longer than twice the filter rise time (Colquhoun and Sigworth, 1983). The idealized trace of each detectable event was superimposed on experimental records for visual inspection. The fraction of time that channels were open was calculated according to the relationship ($\sum$ dwell time $\times$ level no.)/total record time. Open-time distribution which comprises only the duration of single openings were analyzed using the binned (10 bins/decade) maximum likelihood method (Sigworth and Sine, 1987). The data and fitted curves were expressed as square root (events/bin) versus log (open time).

Results

Dynorphins, including dynorphin(1-13), (1-17) and (1-32), block the I_{NMDA} in the same manner (Chen et al., 1995). These dynorphins reduce I_{NMDA} reversibly and the inhibitory action can not be blocked by the opioid antagonist, naloxone, or by the κ -opioid antagonist, nor-BNI (Chen et al., 1995). An example of the time course of dynorphin(1-32) action is shown in Figure 1. Dynorphin(1-32) at 0.25 μ M reduced I_{NMDA} by 60% in this cell. The I_{NMDA} recovered to the control level after the removal of

Figure 2. On and off rates of dynorphin(1-17). To obtain the τ_{on} , I_{NMDA} was recorded during a step application of 2 μ M (A) and 5 μ M dynorphin (B). The holding potential was -70 mV. The periods of solution application are indicated by the solid lines below. Lines drawn through the data points are the theoretical fit of single exponential functions. The zero current lines are indicated by dotted lines. C, To obtain the τ_{off} , the NMDA response was recorded during the rapidly washing out of 5 μ M dynorphin. D, The concentration dependence of $1/\tau$. The SE values larger than the data points are expressed with bars. The τ_{on} values are 1 μ M, 79.2 ± 1.7 msec ($n = 6$); 2 μ M, 57.3 ± 1.8 msec ($n = 6$); 5 μ M, 31.3 ± 1.3 msec ($n = 7$); 8 μ M, 23.3 ± 0.9 msec ($n = 6$); and 10 μ M, 17.8 ± 1.2 msec ($n = 4$). The $1/\tau_{on}$ changed linearly with the concentration of dynorphin and $1/\tau_{off}$ did not change significantly with dynorphin concentration.

dynorphin from the external solution. The same actions of dynorphin could be repeatedly observed in the same cell (Fig. 1).

The dose–response curves for various dynorphins have been examined in detail (Chen et al., 1995). The affinity of dynorphin(1-32) was the highest ($IC_{50} = 0.25$ μ M). The affinities of dynorphin(1-17) ($IC_{50} = 1.6$ μ M) and of dynorphin (1-13) ($IC_{50} = 1.8$ μ M) were similar. The kinetics of the dynorphin(1-17) block were determined using concentration jump experiments (Fig. 2). After the NMDA responses reached a steady state, the external solution was rapidly switched to a solution containing both NMDA and dynorphin (Fig. 2A,B). The decrease in I_{NMDA} was well-fitted by a single exponential; the time constant (τ_{on}) of the binding of dynorphin at 2 μ M was 57.3 ± 1.8 msec ($n = 6$) and at 5 μ M was 31.3 ± 1.3 msec ($n = 6$). The time constant of the unbinding of dynorphin (τ_{off}) was determined as dynorphin was rapidly washed out (Fig. 2C). The τ_{off} at 5 μ M dynorphin(1-17) was 127.0 ± 4.9 msec ($n = 5$). The concentration dependence of $1/\tau$ is shown in Figure 2D. The $1/\tau_{on}$ changed linearly with the concentration of dynorphin. According to a one binding site model, $1/\tau_{on} = k_+ [Dyn] + k_-$ and $1/\tau_{off} = k_-$ where k_+ is the association and k_- the dissociation rate constants of dynorphin, and $[Dyn]$ is the dynorphin concentration. The k_+ obtained from the slope of the curve was 4.9×10^6 sec $^{-1}$ M $^{-1}$. The y-intercept of the curve corresponding to k_- was 7.5 sec $^{-1}$. In contrast, $1/\tau_{off}$ did not change significantly with dynorphin concentration (Fig. 2D). The average $1/\tau_{off}$ (i.e., k_-) obtained from the five concentrations of dynorphin we tested was $k_- = 7.6$ sec $^{-1}$, a value consistent with k_- obtained from the $1/\tau_{on}$ curve. The dissociation constant of dynorphin (K_D) calcu-

Figure 3. Dynorphin speeds up the deactivation of NMDA responses. A, I_{NMDA} recorded after the removal of NMDA from the external solution. The I_{NMDA} was fitted with a double-exponential function. The time constants of the exponentials are 48.0 msec and 338.6 msec. The period of solution application is indicated by the solid line below the trace. The zero current line is indicated by a dotted line. The theoretical curve was drawn through the data points. B, In the presence of 2 μ M dynorphin (1-17), the decay of I_{NMDA} was fitted with a single-exponential function. The time constant is 16.1 msec. C, For better comparison, the two current traces were scaled to show the time constant differences. The holding potential was -70 mV.

lated from on and off rates, that is, $K_D = k_-/k_+$, is 1.6 μ M. This value is the same as the apparent dissociation constants (IC_{50}) of dynorphin(1-17) obtained from the dose–response experiments (Chen et al., 1995). The recovery from the dynorphin (1-32) block occurred much slower than τ_{off} for dynorphin(1-17) (Fig. 1). This is consistent with fact that the affinity of dynorphin(1-32) is about seven times that of dynorphin (1-17).

Since the decay of the NMDA component of excitatory postsynaptic currents (EPSCs) is governed mainly by the deactivation of NMDA receptor channels (Lester et al., 1990; Pan et al., 1993; Jonas and Spruston, 1994), the dynorphin effect on the deactivation can provide clues as to whether dynorphin could change the time course of EPSC. The deactivation of NMDA responses in control and in dynorphin(1-17) (2 μ M) was studied. When the external solution was rapidly switched from an NMDA-containing to an NMDA-free solution, the I_{NMDA} decayed. The decay constants, obtained by fitting the decrease in I_{NMDA} with a double exponential function, were 50 ± 2.8 msec and 428 ± 128 msec ($n = 2$) (Fig. 3A). In the presence of dynorphin, the decay of I_{NMDA} could be fitted with a single exponential function; the decay time constant became 18.4 ± 3.3 msec ($n = 2$). Thus, dynorphin, if released near NMDA receptors, would shorten EPSC, in addition to decreasing its amplitude.

Dynorphins could inhibit NMDA responses by reducing the affinity of NMDA for its recognition site, by changing the binding of glycine or by altering the properties of NMDA receptor channels. To test each of these possibilities, we first determined the dose–response curves for NMDA with and without dynor-

Figure 4. Dynorphin does not act at the NMDA recognition site. *A*, Dose-response curves for NMDA in the control and in 0.25 μM dynorphin(1-32) solutions. Solid lines were drawn according to the equation $I = I_o [NMDA]^h / ([EC_{50}]^h + [NMDA]^h)$. I_o is the maximal current in the control solution, [NMDA] is the concentration of NMDA, and h is the Hill coefficient. The EC_{50} for NMDA was 82.5 μM in control and 78.5 μM in dynorphin. The data are from the same cell. *B*, Effect of dynorphin in different concentrations of glycine solutions. Dynorphin(1-32) at 0.25 μM blocked the I_{NMDA} by 50% in the 100 nM glycine and by 53% in 2 μM glycine.

phin. The EC_{50} for NMDA obtained from fitting the data with the Hill equation (Fig. 4A) was 68.5 ± 9.2 ($n = 7$) in control and 73.3 ± 17.9 ($n = 3$) in 0.25 μM dynorphin(1-32). The change in the EC_{50} was not statistically significant ($p > 0.5$, Student's t test). We further found that the extent of the dynorphin block of I_{NMDA} remained unchanged in different concentrations of external glycine (Fig. 4B). Thus, dynorphin is a non-competitive blocker at the NMDA and at the glycine binding sites.

Unlike Mg^{2+} , dynorphin blocks NMDA responses equally well at various membrane potentials (Chen et al., 1995). Proton (H^+) also inhibits NMDA responses in a voltage-independent manner (Tang et al., 1990; Traynelis and Cull-Candy, 1990; Vyklícký et al., 1990; Traynelis and Cull-Candy, 1991). To test whether dynorphin interacts at the H^+ site, we obtained the dose-response relationship for dynorphin at pH 7.4 (0.04 μM [H^+]) and 6.7 (0.2 μM [H^+]). The NMDA responses were decreased at the low pH (Fig. 5A), similar to those reported previously. However, the IC_{50} of dynorphin was not altered with a fivefold increase in H^+ concentration (Fig. 5B). Thus, it is un-

Figure 5. Interaction of dynorphin with H^+ and Zn^{2+} . *A*, The NMDA-activated currents were reduced by 72% as the pH decreased from 7.4 to 6.7 ($[H^+]$ increased from 0.04 to 0.2 μM). Dynorphin(1-32), at 0.25 μM, inhibited NMDA currents by a similar extent at the two pHs. *B*, The dose-response curves of the dynorphin(1-32) block were recorded at pH = 7.4 and 6.7. The solid lines were drawn using a equation $I = I_o [IC_{50}]^h / ([IC_{50}]^h + [NMDA]^h)$ with $h = 1.0$. The IC_{50} for the dynorphin block was 0.31 μM at pH = 7.4 and 0.32 μM at 6.7. Data were average values from six cells. *C*, The dose-response curves of the Zn^{2+} block were recorded in the control and in 0.25 μM dynorphin(1-32). Data were average values from 10 cells. The solid lines were drawn using a similar equation as in *B* with $h = 1.0$. The IC_{50} for the Zn^{2+} block was 16.3 μM in the control and 14.1 μM in the dynorphin(1-32).

likely that dynorphin binds to the same site as H^+ . Zn^{2+} was found to be released in synaptic clefts (Assaf and Chung, 1984; Howell et al., 1984) and to potently block I_{NMDA} in a noncompetitive and relatively voltage-independent manner (Peters et al., 1987; Westbrook and Mayer, 1987). Dynorphin did not appear

Figure 6. *A*, The effects of DTT and NEM on NMDA responses. The peak I_{NMDA} was recorded at every 2 min interval. The bars indicate the time periods when the cell was treated with 3 mM DTT or 1 mM NEM. The inward I_{NMDA} was slowly increased during DTT application. The NMDA responses remained elevated when NEM was perfused onto the cell immediately after DTT was washed away. *B*, DTT-treated dynorphin blocked NMDA responses equally well. Dynorphin was preincubated in 3 mM DTT solution for 10 min before being applied to the cell. There was no difference in the extent of inhibition of I_{NMDA} by dynorphin and DTT-treated dynorphin. *C*, Effect of DTT on the affinity of dynorphin(1–13) for NMDA receptors. The dynorphin block of NMDA responses was reduced in DTT-treated cells. *Upper*, In the absence of DTT, dynorphin reduced I_{NMDA} by 57%. Following treatment with 3 mM DTT, NMDA responses were increased. Under this condition, dynorphin reduced I_{NMDA} by 27%. The data were obtained from the same cell. The membrane potential was held at -70 mV. *Lower*, The dose–response curves of the dynorphin block before and after DTT treatment. The data are expressed as mean $\pm$ SE for three to eight cells at each dynorphin concentration. The solid lines are the theoretical fit of the average NMDA responses. The IC_{50} of dynorphin was 1.8 μM in control and was 4.8 in DTT-treated cells.

to bind to the same site as Zn^{2+} because the IC_{50} of the dose-dependent block of I_{NMDA} by Zn^{2+} did not change in the presence of dynorphin (Fig. 5C).

The sulfhydryl reducing agent, DTT, was found to potentiate the NMDA responses and to modify the properties of NMDA receptor channels (Aizenman et al., 1989; Lazarewicz et al., 1989; Reynolds et al., 1990; Sucher et al., 1990; Traynelis and Cull-Candy, 1991; Tang and Aizenman, 1993; Kohr et al., 1994). We examined whether dynorphin effects are influenced by the modification of disulfide bonds on the NMDA receptor complex. After treatment with DTT, the amplitude of I_{NMDA} was increased by twofold in our cells (Fig. 6). The potentiation developed slowly. The onset of potentiation was observed 2 min after cells were incubated in DTT solution and the effect reached its steady-state in 10 min (Fig. 6A). When DTT was removed from the external solution, the I_{NMDA} returned to the control level in 6–8 min (data not shown). The potentiating effect of DTT became irreversible when DTT-treated cells were exposed to the alkylation agent *N*-ethylmaleimide (NEM) (Fig. 6A). To ensure that DTT did not alter the properties of dynorphin, we incubated dynorphin first in DTT solution for up to 10 min and then perfused the DTT and dynorphin mixture onto the cells. Because the onset of the DTT effect on NMDA receptors was slow, we were able to test the action of DTT-treated dynorphin prior to the NMDA responses becoming potentiated. There was no difference in the extent of inhibition of I_{NMDA} by dynorphin and by DTT-treated dynorphin (Fig. 6B). We then studied the effect of

dynorphin in DTT-treated cells. Dynorphin became less effective in blocking the NMDA responses (Fig. 6C). The IC_{50} of dynorphin increased from 1.8 μM in control to 4.8 μM in DTT-treated cells (Fig. 6C). Thus reduction of the disulfide bonds located in the NMDA receptor complex significantly changed the binding of dynorphin.

To examine how dynorphin altered the properties of NMDA receptor channels, the effects of dynorphin(1–17) on single NMDA channels were studied in outside-out patches (Fig. 7). The main conductance level did not change in the presence of 1 μM of dynorphin(1–17). The fraction of time channels being open (F) in dynorphin was reduced, thus resulting in a marked decrease in the number of overlapping events (Fig. 7A,B). The NMDA channel activity desensitized and ran down in the continuous presence of the agonist. To find out if the actual decrease was due to the dynorphin block, we measured channel currents in the control solution, during dynorphin (1 μM) application and after washing out dynorphin. An example of the changes in F during each of these periods is given in Figure 7C. The F ratios, that is, $F(\text{dynorphin})/F(\text{control})$ or $F(\text{recovery})/F(\text{control})$, obtained from all the patches are summarized in Figure 7D. The F ratio for dynorphin was $36 \pm 4\%$ ($n = 14$) and for recovery was $64 \pm 9\%$ ($n = 6$). Taking the decay of channel activity into account, dynorphin at 1 μM would reduce channel activities to 56% of the control, which is consistent with the value (61%) obtained in whole-cell experiments (Chen et al., 1995).

To study the effect of dynorphin on the open times of NMDA

Figure 7. Effect of dynorphin(1-17) on single-channel currents. *A*, Single-channel currents were recorded from an outside-out patch at -70 mV before and after dynorphin(1-17) ($1 \mu\text{M}$) treatment. The external solutions contained $10 \mu\text{M}$ NMDA and $2 \mu\text{M}$ glycine. Inward currents appear as downward deflections. *B*, Amplitude histogram obtained from the same patch as shown in *A*. The main current level was 2.6 pA both in the control and in dynorphin. The relative F ratio, that is, $F(\text{dynorphin})/F(\text{control})$, in this patch was 0.22 . *C*, The recovery of dynorphin effect. An example of the time course of F during the control, the dynorphin application and the washing out of dynorphin is shown. Each bar represents F calculated from a 0.4 sec current record. The period of dynorphin application is indicated by the solid line in the graph. *D*, The F ratio relative to that of the control during dynorphin application and recovery period summarized from all the experiments (F for control was set at 100%). The F ratio was $35.6 \pm 4.48\%$ ($n = 14$) for dynorphin ($P < 0.001$, t test) and $64.4 \pm 9.45\%$ ($n = 6$) for recovery ($P > 0.2$).

channels, the channel activities in control and in $1 \mu\text{M}$ dynorphin(1-17) were recorded from the same patches (Fig. 8). The open-time histograms of the main conductance state of NMDA-activated channels were fitted by two exponentials. The fast component was changed from 2.1 ± 1.1 msec in control to 1.4 ± 0.2 msec in dynorphin ($n = 7$); the slow component was decreased from 9.7 ± 0.8 msec to 3.5 ± 0.6 msec. Dynorphin(1-17) reduced the mean open time of NMDA channels by 34% ($p < 0.05$, t test). Because channels opened infrequently in the presence of dynorphin, very low concentrations of NMDA were not used in the single-channel recordings (Jahr and Ste-

vens, 1990; Gibb and Colquhoun, 1992; Lin and Stevens, 1994). Accurate analyses of the closed time could not be obtained with accuracy. Since the decrease of open time was not sufficient to account for the total reduction in whole currents or in F , the closed time of the channel might also be lengthened by dynorphin.

Discussion

Dynorphin reduces NMDA-activated currents without involving opioid receptors (Fig. 1) (Chen et al., 1995). This has led to the suggestion that dynorphin acts directly at the NMDA receptor channels (Chen et al., 1995). We also propose that the dynorphin site is located at the extracellular domain of an NMDA channel because of the large molecular size and the voltage-independent action of dynorphin (Chen et al., 1995). Since dynorphin does not change the EC_{50} of NMDA, and external glycine has no effect on the dynorphin block of NMDA receptor channels, dynorphin does not interact with the NMDA or glycine recognition sites. The facts that the IC_{50} of Zn^{2+} remains the same in the presence of dynorphin and that the IC_{50} of dynorphin is unchanged with increasing H^+ concentration also argue against the interaction of dynorphin with the Zn^{2+} or the H^+ site. Since the effect of dynorphin persists in cell-free patches, second messenger systems do not appear to play a role in the action of dynorphin.

The dynorphin affinity is substantially lowered when the disulfide bonds in NMDA receptors are reduced by DTT. There are at least two possibilities for this to occur: (1) dynorphin interacts with the redox modulatory site and (2) dynorphin acts at a site conformationally linked with the redox sites on the NMDA receptors. Since dynorphin is not an oxidizing agent and the effect of dynorphin, unlike other oxidizing agents, is decreased with DTT treatment, dynorphin does not appear to act directly on the redox site. Our data are consistent with the second possibility, that is, dynorphin binds to a site which is conformationally altered by the sulfhydryl reducing agent. The information on the molecular composition of the NMDA receptors in trigeminal neurons is not yet available. We do not know the subunits on which these redox sites are located. Nevertheless, the redox sites on NR1-NR2A heteromeric channels have been shown to respond to DTT very differently from those on NR1-NR2B, -NR2C, and -NR2D (Kohr et al., 1994). Since the DTT effect in our cells develops slowly and can be made irreversible by the alkylation agent NEM, the redox sites associated with dynorphin are not likely to be on the NR2A subunit. In the spinal dorsal horn, NR1 mRNA is abundantly expressed, with NR2D lightly expressed and with NR2C occasionally found in lamina II neurons (Tolle et al., 1993). NR2A and NR2B mRNA are not detectable (Tolle et al., 1993). Our DTT effects are consistent with the redox sites on the NR1-NR2C or NR1-NR2D subunits. Single-channel analyses indicate that dynorphin reduces the mean open-time of NMDA receptor channels. The decrease in F can account for the reduction in whole-cell currents. Perhaps, dynorphin acts at a site conformationally linked with the redox modulatory site on NR1-NR2C or NR1-NR2D subunits at the extracellular domain of the NMDA receptor channel. In any case, the reduction of dynorphin effect after the DTT modification of NMDA receptor channels further supports our contention that dynorphin acts directly on NMDA receptors, thus altering the conformation of channel protein and thereby changing the gating property of the channel.

At first glance, the apparent dissociation constants of dynor-

Figure 8. Open-time histograms in control and in $1\ \mu\text{M}$ dynorphin(1-17). The open-time distributions for NMDA receptor channels were binned logarithmically. The events/bin is expressed as a square root scale. The histograms were fitted by the sum of two exponentials (continuous line). The time constants were indicated by arrows. The mean open times in control were 1.8 and 10.5 msec; the relative areas associated with the two exponentials were 0.97 and 0.03. The mean open times in dynorphin were 1.2 and 3.8 msec; the relative areas associated with each exponential were 0.98 and 0.02.

phin for NMDA receptors seem rather high, considering the dynorphin level in wet tissue is 30–50 nM in the brain and in the spinal cord (Millan et al., 1986). However, the action of a transmitter is determined by its concentration in the synaptic cleft, which is usually several orders of magnitude higher than that in the extracellular space. For example, glutamate concentration was estimated to be 100 mM in the synaptic vesicle (Nicholls and Attwell, 1990; Kanai et al., 1993), 1 mM at the postsynaptic membrane (Clements et al., 1992) and $1\ \mu\text{M}$ in the extracellular space (Nicholls and Attwell, 1990; Kanai et al., 1993). Various forms of enkephalins, dynorphins and their precursors were found to release from nerve terminals (Chavkin et al., 1983; Terrian et al., 1990) or to co-release with catecholamine from chromaffin granules and dense cored vesicles of the sympathetic nerve (Wilson et al., 1980; Dumont et al., 1983; Rossier et al., 1984). The estimated concentration of enkephalin in the vesicles was between 0.4 and 1.8 mM (Viveros et al., 1979; Wilson et al., 1980; Winkler and Wethead, 1980; Klein et al., 1982). The dynorphin content was seven to eight times less than the enkephalin content (Klein and Thureson-Klein, 1984; Millan et al., 1986). Assuming the average width of excitatory synapses to be 20 nm and the diameter of synapses to be around $2\ \mu\text{m}$ (Eccles and Jaeger, 1958), the simple diffusion model predicts that the concentration of dynorphin at the NMDA receptor in the postsynaptic membrane would reach tens of μM , a value within the range observed for IC_{50} of dynorphin. Furthermore, the dynorphin level increases two- to fourfold with tissue injury. Thus, under physiological and pathological conditions, dynorphin released in response to high frequency stimulation in the vicinity of synaptic clefts would reach a sufficiently high concentration to affect the activity of NMDA receptor channels.

The direct block of NMDA responses may well be a general action of dynorphin, since the same dynorphin effect has been observed in isolated periaqueductal gray neurons (Lai and Huang, unpublished observations). If dynorphin-containing terminals can modulate neuronal activity without the involvement

of opioid receptors, this may explain the prevalence of the naloxone-insensitive actions of dynorphin (Moise and Walker, 1985; Faden, 1990; Dubner and Ruda, 1992; Caudle et al., 1994) and the mismatch between the distribution of opioid receptors and opioid-containing terminals (Mansour et al., 1988).

Since dynorphin reduces glutamate release (Wagner et al., 1993; Weisskopf et al., 1993) and blocks NMDA responses (Fig. 1; Chen et al., 1995), the net effect of dynorphin at NMDA receptors is inhibitory. Therefore, dynorphin can not sensitize nociceptive projection neurons by direct interaction on NMDA receptors as previously proposed (Dubner and Ruda, 1992). However, this does not exclude the possibility that dynorphin exerts excitatory action on projection neurons through a disinhibition mechanism(s) (Zieglansberger and Tulloch, 1979; Madison and Nicoll, 1988). This could occur when dynorphin reduces the activity of GABA- or glycine-containing interneurons, resulting in an overall loss of inhibitory control of the projection neurons. The dynorphin action described here may also be important in modulating neuronal activity in other regions of the brain. For instance, in the CA3 hippocampus, one of the effects of dynorphin(1-17), which is not opioid receptor mediated, is to enhance NMDA synaptic currents (Caudle et al., 1994). Although the functional role of this dynorphin effect has yet to be elucidated, it would be interesting to determine whether disinhibition also plays a role in this dynorphin action and if dynorphin contributes to the development of activity-dependent neuronal plasticity such as long term potentiation (Ben-Ari et al., 1992; Bliss and Collingridge, 1993) in hippocampal neurons.

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