κ_2 Opioid Receptors Inhibit NMDA Receptor-mediated Synaptic Currents in Guinea Pig CA3 Pyramidal Cells

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The role of the endogenous opioid peptide dynorphin (1-17) in regulating NMDA receptor-mediated synaptic currents was examined in guinea pig hippocampus. Schaffer collateral/commissural fiber-evoked NMDA synaptic currents were recorded using whole-cell patch-clamp techniques in CA3 pyramidal cells. Dynorphin was found to have dual effects on NMDA synaptic currents, increasing currents at low concentrations and decreasing currents at high concentrations. Only the inhibitory action of dynorphin was sensitive to naloxone, indicating that this effect was mediated by an opioid receptor. The inhibitory effect was mimicked by bremazocine, but not by U69,593, U50,488, [D-Ala², N-Me-Phe⁴, Gly-ol]-enkephalin, or [D-Pen^{2,5}]-enkephalin. Bremazocine's effect was blocked by naloxone, but not by nor-binaltorphimine, cyprodime, or naltrindole. These findings suggest that bremazocine's effect was mediated by the κ_2 subtype of opioid receptor. In addition, 1 μ M naloxone and antisera to dynorphin (1-17) were found to increase NMDA-mediated synaptic currents. Nor-binaltorphimine, cyprodime, naltrindole, and antisera to met-enkephalin did not increase the NMDA synaptic current. These findings suggest that endogenous dynorphin was acting at κ_2 receptors to inhibit NMDA receptor-mediated synaptic currents. Overall, these findings indicate that dynorphin is an endogenous agonist for κ_2 receptors in the CA3 region of the guinea pig hippocampus and that these receptors regulate NMDA receptor function.

[Key words: opioids, dynorphin, kappa receptors, N-methyl-D-aspartate receptors (NMDA), whole cell patch clamp, hippocampus, guinea pig, CA3]

The opioid peptide dynorphin is thought to be the endogenous ligand for κ -opioid receptors (Chavkin et al., 1982). This peptide has been examined in numerous models in order to determine its functional role in the CNS. The effects produced by exogenously applied dynorphin vary from neurotoxicity (Caudle and Isaac, 1987) to inhibition of calcium potentials (Werz and MacDonald, 1985; MacDonald and Werz, 1986; Gross and MacDonald, 1987). These differing actions of exogenous dynorphin have not provided a clear answer to dynorphin's function in the CNS. Adding to the confusion are reports demonstrating that

dynorphin's concentration in the spinal cord increases following peripheral tissue and nerve injury (Iadarola et al., 1988; Nahin et al., 1989; Kajander et al., 1990), and spinal cord injury (Faden et al., 1985), and that its concentration in the hippocampus increases (Lee et al., 1987) and decreases (McGinty et al., 1986) following seizures. These studies of endogenous dynorphin have also provided little information concerning dynorphin's role in the CNS. In our present course of investigation, we have narrowed our studies to structures that have dynorphin-containing afferents that were demonstrated to release dynorphin under physiological conditions. The logic behind this approach is that an effect of exogenous dynorphin should be demonstrable for endogenous dynorphin. Thus, if exogenous and endogenous dynorphin produce similar effects it is likely that a physiological function for dynorphin was demonstrated.

The hippocampus of rodents contains a substantial concentration of dynorphin. This dynorphin resides primarily in the mossy fiber pathway that extends from the dentate granule cells to the CA3 pyramidal cells (Gall et al., 1981; McGinty et al., 1983; McLean et al., 1987). The mossy fibers serve as the major excitatory input to the CA3 region. Previous attempts to demonstrate effects of dynorphin and other κ -selective opioids in the CA3 region of the rodent hippocampus were either unsuccessful (Caudle and Chavkin, 1990) or ambiguous (Moises and Walker, 1985). On the other hand, we demonstrated that κ agonists, including dynorphin, inhibit excitatory transmitter release from the perforant path onto dentate granule cells in guinea pig hippocampus (Wagner et al., 1992, 1993). Other groups have found that dynorphin, through κ receptors, inhibits calcium currents and calcium action potentials in cultured dorsal root ganglion cells (Werz and MacDonald, 1985; MacDonald and Werz, 1986; Gross and MacDonald, 1987). Therefore, we proposed that the inhibition of calcium currents was likely to be the mechanism for κ receptor-mediated inhibition of excitatory amino acid release from the perforant path (Wagner et al., 1992, 1993).

Using the κ_1 -selective ligand ³H-U69,593 for autoradiography, we found that virtually all of the specific binding was located in the molecular layer of the guinea pig dentate gyrus (Wagner et al., 1991, 1992). This pattern of binding by ³H-U69,593 explained the lack of effect of this compound in the CA3 region of the guinea pig hippocampus in our physiological studies (Caudle and Chavkin, 1990). However, Weisskopf et al. (1993) reported that U69,593 had inhibitory effects in the guinea pig CA3. Given the lack of binding sites for U69,593 in this region of the guinea pig hippocampus, Weisskopf et al.'s findings are difficult to interpret.

Recently, evidence was presented indicating the existence of at least two subtypes of κ -opioid receptors (Nock et al., 1988,

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1990, 1993; Zukin et al., 1988; Rothman et al., 1992). U69,593 selectively binds to one of these sites and this site has been labeled the κ_1 receptor. Compounds like ethylketocyclazocine and bremazocine, in the presence of μ - and δ -opioid receptor blockers, bind to the U69,593 binding site and to another site with approximately equal affinity. The U69,593-insensitive site has been labeled the κ_2 receptor (Zukin et al., 1988; Rothman et al., 1992) or the ϵ receptor (Nock et al., 1990, 1993). Autoradiographic studies of κ -opioid binding sites using either ^3H -ethylketocyclazocine or ^3H -bremazocine in guinea pig hippocampus resulted in binding to both the molecular layer of the dentate gyrus and the stratum lucidum of the CA3 (McLean et al., 1986; Zukin et al., 1988). The binding in the CA3 contrasts with ^3H -U69,593 binding (Wagner et al., 1991, 1992), and suggests that these sites are κ_2 or ϵ receptors.

The close proximity of the κ_2 binding sites in the guinea pig CA3 to the terminals of the dynorphin-containing mossy fibers, as compared to the κ_1 binding sites in the dentate gyrus, suggests that dynorphin may be an endogenous ligand for this receptor. If endogenous dynorphin acts at κ_2 receptors, the question then becomes what physiological function do κ_2 receptors perform and how can we measure that function? Several studies have suggested that dynorphin may play a role in the regulation of NMDA receptors (Caudle and Isaac, 1988a,b; Bakshi and Faden, 1990a,b; Faden et al., 1990; Isaac et al., 1990; Shukla et al., 1992; Skilling et al., 1992). In these studies, selective antagonists to the NMDA receptor complex blocked the effects of exogenously applied dynorphin. The results of these experiments suggest that dynorphin may potentiate NMDA receptor function. However, several other studies have demonstrated that dynorphin has inhibitory actions in the CNS. In addition to the κ_1 inhibitory effects, several investigators have found neuroprotective (Baskin et al., 1984; Garant et al., 1985), inhibitory (Stevens et al., 1987; Stewart and Isaac, 1991), and both excitatory and inhibitory effects of dynorphin (Moises and Walker, 1985; Knox and Dickenson, 1987; Hylden et al., 1991) that may or may not be mediated by κ -opioid receptors. The neuroprotective effects of dynorphin (Baskin et al., 1984; Garant et al., 1985) are similar to those of NMDA receptor antagonists (Choi et al., 1988), suggesting that dynorphin may act, in the neuroprotection experiments, by inhibiting the NMDA receptor complex.

In addition to dynorphin's actions at opioid receptors, it was found to have nonopioid actions as well (Faden and Jacobs, 1984; Moises and Walker, 1985). Supporting this finding are receptor binding studies by Massardier and Hunt (1989) and Shukla et al. (1992) demonstrating that dynorphin inhibits binding of ^3H -glutamate and ^3H -MK-801 to NMDA binding sites in membranes. This effect of dynorphin was not sensitive to opioid antagonists, implying that the dynorphin was not acting at an opioid receptor. The inhibition of the binding of these agents at NMDA receptors by dynorphin, rather than the enhancement of binding, is not entirely consistent with the excitatory effects of dynorphin, but is compatible with the neuroprotective actions of dynorphin (Baskin et al., 1984; Garant et al., 1985). However, until physiological studies are performed the interpretation of the binding studies remains difficult.

If κ_1 receptors mediate inhibition of the voltage-sensitive calcium currents, then perhaps κ_2 opioid receptors and the "non-opioid dynorphin receptors" mediate the excitatory and inhibitory actions of dynorphin that are thought to be mediated through the NMDA receptor complex. In order to study κ_2 re-

Figure 1. Diagrammatic representation of a guinea pig hippocampal slice. The cell that the patch electrode is connected to is a CA3 pyramidal cell. The stimulating electrode is placed in the Schaffer collateral/commissural pathway. The gray shaded area represents the location of NMDA receptors in the CA3 region of the guinea pig hippocampus. The hatched area represents the location of U69,593-insensitive bremazocine binding sites (κ_2 receptors) in the guinea pig hippocampus.

ceptor physiology and dynorphin's effects on κ_2 receptors, it was necessary to dissect κ_1 , κ_2 , and nonopioid effects from each other. To avoid the influence of κ_1 receptors, the CA3 region of the guinea pig hippocampus was chosen for study. As previously mentioned, this region of the guinea pig hippocampus lacks ^3H -U69,593 binding sites (Wagner et al., 1992) and is innervated by a dynorphinergic fiber bundle (Gall et al., 1981; McGinty et al., 1983; McLean et al., 1987). Using whole-cell voltage-clamp techniques, we demonstrate that κ_2 receptors inhibit NMDA-mediated synaptic currents in the CA3 region of the guinea pig hippocampus, and evidence is presented that endogenous dynorphin acts at these receptors.

Materials and Methods

Electrophysiology. Hippocampal slices were prepared from 150–300 gm male Hartley guinea pigs as previously described (Caudle and Chavkin, 1990). Briefly, the guinea pigs were anesthetized with pentobarbital (50 mg/kg, i.p.) and then decapitated. The brains were removed and chilled in ice-cold Krebs's bicarbonate buffer of the following composition (mM): NaCl (124), KCl (4.9), KH_2PO_4 (1.2), MgSO_4 (2.4), CaCl_2 (2.5), NaHCO_3 (25.6), and glucose (10). The brains were then sliced on a vibratome (500 μm). The hippocampal slices were dissected from the rest of the tissue and placed in a tissue chamber. The slices were then superfused (1 ml/min) with Krebs's bicarbonate buffer. The temperature was maintained at 34°C and the buffer was bubbled with 95% O_2 /5% CO_2 . The slices were allowed to equilibrate in the chamber at 34°C for a minimum of 1 hr before experiments were started to ensure that residual pentobarbital was washed out of the tissue. For recording NMDA receptor-mediated currents, the superfusion buffer was changed to Krebs's bicarbonate buffer with nominally zero magnesium, 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and 20 μM bicuculline. A concentric bipolar stimulating electrode (SNE 100, Rhodes Medical Instruments, Woodland Hills, CA) was placed in the stratum lacunosum moleculare (Schaffer collaterals) of the hippocampal slice, approximately at the CA1/CA3 border (Fig. 1). Stimuli consisted of single 0.3 msec square waves with currents ranging from 200 to 400 μA . Patch-clamp electrodes were pulled to resistances of between 2 and 10 M Ω . The electrodes were filled with (mM) CsCl (120), tetraethylammonium chloride (20), CaCl_2 (1), MgCl_2 (2), EGTA (10), HEPES (10), ATP (4), and GTP (0.5), and the pH was adjusted to 7.4 with CsOH. All data was collected using an Axopatch 200 (Axon Instruments, Foster City, CA). The data was digitized and recorded on a personal computer for future analysis.

A.

Figure 2. *A*, Representative Schaffer collateral/commissural fiber-evoked NMDA receptor-mediated current in a guinea pig CA3 pyramidal cell. The current was measured in the absence of magnesium and in the presence of the non-NMDA excitatory amino acid antagonist CNQX ($10 \mu\text{M}$) and the GABA antagonist bicuculline ($20 \mu\text{M}$). In addition, the electrode contained cesium and tetraethylammonium chloride to block potassium channels. The holding potential while recording synaptic currents was $+5 \text{ mV}$. Depolarizing the cells inactivated voltage-gated sodium and calcium currents and reduced the amplitude of the NMDA-mediated synaptic current. As illustrated by the traces, the current was blocked by the selective NMDA antagonist APV ($50 \mu\text{M}$). When the antagonist was washed out of the bath the current returned. Each trace represents an average of five sweeps. The total length of the sweep was 960 msec. *B*, Dynorphin (1-17) concentration-response relationship on NMDA receptor-mediated currents. The area over the curve was calculated for traces similar to those in *A* to determine the amount of charge (current multiplied by time) that entered the cells during the recordings. The charge entering the cell in the presence of dynorphin was then compared to the charge prior to dynorphin (Control) to determine the percent of control value. Each point represents between four and nine cells. Error bars represent SEM.

B.

Experimental procedure. Guinea pig CA3 pyramidal cells were voltage clamped and held at -65 mV . When the cell had stabilized the Krebs's bicarbonate buffer superfusing the slices was changed to buffer that was the same in all respects except that it contained nominally zero magnesium, $10 \mu\text{M}$ CNQX, and $20 \mu\text{M}$ bicuculline. Bicuculline was used to block GABA_A-mediated currents and CNQX was used to block non-NMDA excitatory amino acid-mediated currents. The electrode contained tetraethylammonium chloride and cesium to block both GABA_B and voltage-sensitive potassium currents. The Schaffer collateral/commissural pathway was chosen for stimulation because the mossy fiber/CA3 pyramidal cell synapse lacks NMDA receptor-mediated currents (Griffith, 1990) (see Fig. 1). Prior to stimulating the Schaffer collateral/commissural pathway the cell was stepped to $+5 \text{ mV}$ and held there for 6 sec. The cells were depolarized for two reasons. First, depolarization was necessary to inactivate voltage-gated sodium and calcium channels that, if left active, seriously contaminate the NMDA receptor-mediated synaptic current. The complete inactivation of these currents was evidenced by the holding current becoming steady after a few seconds. Second, the cells were depolarized to reduce the amplitude of the NMDA receptor-mediated current. The peak NMDA synaptic currents when the cells were held at $+5 \text{ mV}$ were between 100 pA and 2 nA. At -65 mV the peak currents were typically in excess of 2 nA. The smaller currents reduce the influx of calcium and decrease the likelihood of

calcium-mediated injury to the cells. The Schaffer collateral/commissural pathway was then stimulated and the synaptic current was recorded. Recording began 60 msec prior to the stimulation and continued for 900 msec after the stimulus. The stimulus intensity was adjusted to achieve the maximal synaptic current with the lowest possible stimulus, generally between 200 and 400 μA . The rate of stimulation never exceeded one every 30 sec in order to prevent long-term potentiation. In addition, the synaptic current had to be stable for 15 min prior to the addition of any drugs to the bathing solution.

Data recorded from the NMDA-mediated synaptic currents are presented as charge entering the cell (current multiplied by time). Charge is used, rather than peak current, because the amount of charge entering the cell determines how far the cell will be depolarized by the opening of the channels; the more charge entering the cell, the more depolarized the cell would become if the cell were not voltage clamped. Thus, the total charge entering the cell represents a more reliable measure of channel activity than peak current.

Statistical analysis of data consisted of unpaired *t* tests, paired *t* tests, and ANOVAs when appropriate; $p < 0.05$ was used as the criteria for significance.

Drugs. Dynorphin (1-17) was purchased from Sigma (St. Louis, MO) and Peninsula Laboratories (Belmont, CA). Bicuculline methiodide, ATP, GTP, and naloxone were purchased from Sigma (St. Louis, MO).

Rabbit antisera to met-enkephalin, rabbit antisera to dynorphin (1-17), and [D-Ala², N-Me-Phe⁴, Gly-ol]-enkephalin (DAMGO) were purchased from Peninsula Laboratories (Belmont, CA). U69,593, U50,488, bremazocine, nor-binaltorphimine (NorBNI), 2-amino-5-phosphonovalerate (APV), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), [D-Pen^{2,3}]-enkephalin (DPDPE), cyprodime, and naltrindole were purchased from Research Biochemicals Inc. (Natick, MA). All drugs were dissolved and applied in the superfusion buffer. All drugs were superfused over the hippocampal slices for at least 10 min before measurements of synaptic responses were made.

Results

Guinea pig CA3 pyramidal cells were voltage clamped as described in Materials and Methods, and NMDA receptor-mediated synaptic currents were recorded. The average charge (current multiplied by time) moving into the pyramidal cells as a result of a maximal stimulus to the Schaffer collateral/commissural pathway was $(51.3 \pm 8) \times 10^{-11}$ C (mean $\pm$ SE, $N = 21$). This current was blocked by the selective NMDA antagonist 2-amino-5-phosphonovalerate (APV) (Fig. 2A; $N = 3$) and was stable for several hours in most cells.

After collecting baseline currents, various concentrations of dynorphin (1-17) were superfused in the buffer and the effect of dynorphin on the NMDA-mediated current was determined. As illustrated by Figure 2B, dynorphin at concentrations between 10 nM and 1 μ M increased the NMDA-mediated current. However, at a concentration of 5 μ M dynorphin, the highest concentration tested, the current was inhibited. The holding current for the step to +5 mV was not altered by dynorphin: 209 ± 60 pA before the addition of dynorphin and 218 ± 70 pA in the presence of 5 μ M dynorphin (mean $\pm$ SE, $N = 5$). The similar holding currents indicate the change in synaptic current was not likely to be due to alterations in membrane conductance.

If 1 μ M naloxone was included in the buffer prior to and during the addition of dynorphin, 5 μ M dynorphin increased the current (Fig. 3), and again the holding current at +5 mV was not altered by the drugs (199 ± 110 pA, $N = 3$). The combination of 1 μ M naloxone and 5 μ M dynorphin not only increased the NMDA-mediated current, but also reduced the survival time of the cells to less than 30 min following the addition of these drugs to the bath ($N = 3$). In our hands, most pyramidal cells that were not exposed to this combination of drugs survived until the electrode was manually pulled from them. Although this observation was not quantified, the toxicity of overstimulated NMDA receptors is well documented (Choi et al., 1988) and any enhancement of their activity is expected to be associated with increased cell mortality. One likely consequence of this toxicity was that the current measured in the presence of 1 μ M naloxone and 5 μ M dynorphin was underestimated due to the cells being damaged prior to recording the synaptic currents. Naloxone had no effect on the excitatory actions of lower concentrations of dynorphin (Fig. 3).

The biphasic nature of the dose-response relationship indicated that dynorphin (1-17) had both an excitatory and an inhibitory action on NMDA currents. These effects could be separated from each other by the concentration of dynorphin and by sensitivity to naloxone. The inhibitory action of dynorphin was sensitive to naloxone, suggesting that this may be an effect at κ -opioid receptors. The excitatory effect of dynorphin was not sensitive to naloxone, indicating that this effect was nonopioid in nature and may be the result of dynorphin binding directly to the NMDA receptor complex (see Massardier and Hunt, 1989; Shukla et al., 1992). The nonopioid effect was not studied further.

Figure 3. Antagonism of dynorphin (1-17)'s effects by naloxone. Data were collected as described in Figure 2 for 5 μ M dynorphin and 100 nM dynorphin in the presence and absence of 1 μ M naloxone. The traces at the top are sweeps from representative cells for each condition. Each trace represents the average of five sweeps. The control responses for the dynorphin + naloxone-treated cells were determined in the presence of 1 μ M naloxone. Paired t tests were used on the raw charge data to compare control currents to currents in the presence of dynorphin. An asterisk indicates that the dynorphin current was significantly different from its control ($p < 0.05$). Bars represent the means of three to nine cells. Error bars represent SEM.

To determine what opioid receptor subtype mediated the inhibitory effect of dynorphin on synaptically mediated NMDA currents, a series of opioid agonists were tested. The selective κ_1 agonists U69,593 (1 μ M) and U50,488 (500 nM) (Nock et al., 1988, 1990, 1993; Zukin et al., 1988; Rothman et al., 1992) had no effect on the NMDA-mediated current (Fig. 4). This finding is consistent with the lack of κ_1 binding sites in the CA3 region of the guinea pig hippocampus (Wagner et al., 1992). The selective μ -opioid receptor agonist [D-Ala², N-Me-Phe⁴, Gly-ol]-enkephalin (DAMGO, 1 μ M) increased the NMDA current, as did the selective δ -opioid receptor agonist [D-Pen^{2,3}]-enkephalin (DPDPE, 1 μ M) (Fig. 4). The viability of the cells did not appear to be compromised by the excitatory actions of DAMGO. However, the cells were clearly injured by 1 μ M DPDPE in a manner similar to when the 1 μ M naloxone/5 μ M dynorphin combination was applied. Because of the injury to the cells, the increase in the NMDA-mediated current as a result of DPDPE was likely to be underestimated. Findings similar to these for DAMGO were reported previously (Chen and Huang, 1991).

Figure 4. Effect of various opioid agonists on NMDA receptor-mediated currents. Data were collected as described in Figure 2. Paired *t* tests were performed on the raw charge data to compare NMDA currents without the opioid agonist to the currents in the presence of the opioid. Bars represent means from three to six cells. An asterisk indicates that the current in the presence of the opioid was significantly different from its control ($p < 0.05$). Error bars represent SEM. The traces at the bottom are from a representative cell and demonstrate the inhibition of the NMDA-mediated current by bremazocine. Each trace represents the average of five sweeps.

The relatively nonselective κ agonists bremazocine (1 μ M) and 5 μ M dynorphin (1-17) inhibited the NMDA-mediated synaptic current (Fig. 4). The holding current at +5 mV was not affected by bremazocine (102 $\pm$ 27 pA before bremazocine, 82 $\pm$ 16 pA in the presence of bremazocine, mean $\pm$ SE, $N = 5$). The inhibitory effects of bremazocine and dynorphin and the lack of inhibitory effects of U69,593, U50,488, DAMGO, and DPDPE suggested that the receptor mediating the inhibition of the NMDA synaptic current was likely to be the κ_2 opioid receptor (see Nock et al., 1990, 1993).

To determine whether bremazocine was acting as a selective κ_2 agonist in this assay, concentration–response relationships in the presence and absence of 100 nM naloxone were determined. As illustrated by Figure 5A, 100 nM naloxone shifted bremazocine's concentration–response relationship to the right. Using the equation $K_i = [\text{antagonist}] / (EC_{50}' / EC_{50} - 1)$, where EC_{50}' is the EC_{50} of bremazocine in the presence of naloxone (Kosterlitz and Watt, 1968), a K_i of 7.5 nM was calculated for naloxone from the data in Figure 5A. This finding is consistent with K_i values reported for naloxone at κ_2 binding sites that range from

A.

B.

Figure 5. A, Bremazocine concentration–response relationships. Data were collected as previously described for various concentrations of bremazocine. The experiments were then performed in the presence of 100 nM naloxone. Each point represents between three and six cells. Error bars represent SEM. The lines were significantly different ($p < 0.001$, ANOVA). B, Antagonism of bremazocine's inhibition of NMDA-mediated synaptic currents with opioid antagonists. Data were collected as previously described. For the experiments with the antagonists, the antagonist was included in the buffer during the control measurements. Each bar represents the mean of three to six cells. Asterisk indicates $p < 0.05$ when compared to 1 μ M bremazocine by unpaired *t* test. Error bars represent SEM.

7 to 10.5 nM (Rothman et al., 1992; Nock et al., 1993; Webster et al., 1993).

The inhibitory effects of 1 μ M bremazocine were completely blocked by 1 μ M naloxone, but not by the κ_1 -selective antagonist nor-binaltorphimine (NorBNI) (Fig. 5B). This finding is consistent with binding studies indicating that NorBNI has little affinity for κ_2 binding sites (Nock et al., 1988, 1990, 1993; Zukin et al., 1988; Rothman et al., 1992). In addition, the μ -selective antagonist cyprodime (Schmidhammer et al., 1990) and the δ -selective antagonist naltrindole (Contreras et al., 1993) also had no effect on the inhibitory actions of bremazocine (Fig. 5B). The results of these opioid antagonist studies and the previous opioid agonist studies indicate that bremazocine acts as a selective κ_2 agonist in this assay.

One observation that was made early in these investigations was that 1 μ M naloxone produced an increase in the NMDA-

mediated synaptic current (Fig. 6A). The holding current at +5 mV was not altered by naloxone: 186 ± 26 pA for cells not exposed to naloxone (mean $\pm$ SE, $N = 21$) and 176 ± 74 pA for cells in the presence of $1 \mu\text{M}$ naloxone ($N = 9$). The opioid antagonists NorBNI, cyprodime, and naltrindole did not influence the NMDA current. The currents were $95 \pm 3\%$, $136 \pm 20\%$, and $93 \pm 10\%$ of control, respectively. Because of the excitatory effect of naloxone, all of the antagonist experiments discussed above compared the currents measured in the presence of antagonist to currents recorded in the presence of both the antagonist and agonist. The excitatory effect of naloxone suggested that an endogenous agonist may be acting on the κ_2 receptors. As described in Materials and Methods, all recordings of NMDA synaptic currents were made in nominally zero magnesium. This low-magnesium buffer results in substantial spontaneous activity in the hippocampal slice; thus, it was likely that endogenous peptides were released continuously during the experiment. Because the mossy fibers contain dynorphin and terminate near the κ_2 receptors, and because we found dynorphin to be an agonist for κ_2 receptors, we hypothesized that dynorphin was the endogenous agonist. To test this hypothesis, rabbit antisera to dynorphin (1-17) or antisera to met-enkephalin were added to the superfusion buffer at a dilution of 1:1000. Figure 6B shows that the antisera to dynorphin increased the NMDA-mediated current, while the antisera to met-enkephalin did not. The increase in the NMDA synaptic current was similar to that produced by naloxone, suggesting that dynorphin, or a dynorphin-like peptide, was being released by the spontaneous activity in the slice and that it acted upon κ_2 receptors.

Previous work demonstrated that κ_1 receptors inhibited excitatory amino acid release presynaptically (Wagner et al., 1992, 1993). The data presented above are entirely consistent with a presynaptic site for the κ_2 receptors; therefore, it was important to determine if excitatory amino acid release was altered by κ_2 receptor activation. To examine this question, the NMDA receptor antagonist APV ($50 \mu\text{M}$) was substituted for the non-NMDA excitatory amino acid receptor antagonist CNQX in the superfusion buffer. This substitution allowed the measurement of non-NMDA excitatory amino acid synaptic currents using the same protocol as was used for NMDA synaptic currents. Presumably, NMDA and non-NMDA excitatory amino acid receptors at the Schaffer collateral/CA3 synapse receive excitatory amino acids from the same axon terminals. Thus, if the synaptic currents exhibit different pharmacologies, the effect most probably occurs postsynaptically rather than through a change in the release of excitatory amino acids presynaptically. Figure 7A illustrates a typical non-NMDA excitatory amino acid-mediated current and its sensitivity to CNQX. The average charge entering the cell at +5 mV following stimulation of the Schaffer collateral/commissural fibers was $(11.2 \pm 4) \times 10^{-11}$ C (mean $\pm$ SE, $N = 9$). As Figure 7B demonstrates, $5 \mu\text{M}$ dynorphin (1-17) had no effect on the non-NMDA excitatory amino acid synaptic current, while $1 \mu\text{M}$ naloxone inhibited the current. The lack of effect of dynorphin and the inhibitory effect of naloxone on non-NMDA-mediated synaptic currents indicated that alterations in the release of excitatory amino acids were not responsible for the effects of these agents on NMDA-mediated synaptic currents. Therefore, we concluded that the κ_2 receptors were likely to be situated postsynaptic to the Schaffer collateral terminals. The κ_2 receptors may reside on the CA3 pyramidal cells; however, further work will be needed to confirm this hypothesis.

Figure 6. *A*, Effect of naloxone on NMDA-mediated synaptic currents. NMDA-mediated currents were collected in the absence of any opioid antagonist (Control) ($N = 21$) and in the presence of $1 \mu\text{M}$ naloxone ($N = 9$). Representative traces are presented at the top. Each trace represents the average of five sweeps. The total charge entering the cells during the recording was then calculated and the means plotted. Asterisk indicates $p < 0.05$ when compared to control by unpaired t test. Error bars represent SEM. *B*, Effect of antisera to opioid peptides on NMDA-mediated synaptic current. Synaptic currents were collected in the absence and presence of either antisera to met-enkephalin ($N = 4$) or antisera to dynorphin (1-17) ($N = 5$). Paired t tests were performed on the raw charge data comparing the charge before and after the addition of the antisera to the bathing solution. Asterisk indicates $p < 0.05$. Error bars represent SEM.

Discussion

Previous studies demonstrated that dynorphin had both opioid and nonopioid effects (Faden and Jacobs, 1984; Moises and Walker, 1985) that were difficult to separate. These opioid and nonopioid effects were again demonstrated in this study; however, we were able to separate these effects by altering the concentration of dynorphin and by the use of naloxone. Our major finding was that a receptor that bears similar pharmacology to that reported for the κ_2/ϵ -opioid binding site (Nock et al., 1988, 1990, 1993; Zukin et al., 1988; Rothman et al., 1992) inhibits

Figure 7. *A*, Representative Schaffer collateral/commissural fiber-evoked non-NMDA excitatory amino acid-mediated synaptic current. The same protocol was used as for NMDA-mediated currents described in Figure 2*A* except that the non-NMDA excitatory amino acid antagonist CNQX was replaced by 50 μ M APV. The traces demonstrate that 20 μ M CNQX blocks the current and that upon removal of CNQX from the superfusion solution the current returns. Traces represent the average of five sweeps. The length of the trace was 960 msec. *B*, Effect of naloxone ($N = 5$) and 5 μ M dynorphin (1–17) ($N = 4$) on the non-NMDA excitatory amino acid-mediated current. Data were collected as previously described. Paired *t* tests were performed on the raw charge data comparing the responses in the absence and presence of drug. Asterisk indicates $p < 0.05$. Error bars represent SEM.

NMDA receptor-mediated synaptic currents. The data supporting this claim are threefold. First, the only opioid agonists tested that inhibit the NMDA receptor-mediated synaptic current are bremazocine and dynorphin. The κ_1 -selective agonists U69,593 and U50,488 did not influence the NMDA receptor-mediated current. The selective μ agonist DAMGO and the selective δ agonist DPDPE enhanced rather than inhibited the current. Concentrations of these opioid agonists above 1 μ M were not tested in this study because in previous work we found that 1 μ M of these agents usually produces a maximum effect (Caudle and Chavkin, 1990; Wagner et al., 1991, 1992, 1993). In addition, opioid receptor selectivity for these agonists begins to break down at concentrations above 1 μ M. This pattern of agonist selectivity is consistent with binding studies describing the κ_2 receptor (Nock et al., 1988, 1990, 1993; Zukin et al., 1988; Rothman et al., 1992). In addition, the relative potencies of bremazocine and dynorphin at inhibiting the NMDA current (bremazocine $\gg$ dynorphin) are consistent with their binding affinities for the κ_2 receptor (Nock et al., 1988, 1990, 1993; Zukin

et al., 1988; Rothman et al., 1992). However, the nonopioid excitatory effect of dynorphin probably antagonized the inhibitory opioid effect, resulting in a reduction in dynorphin's potency. Second, the inhibitory effect of bremazocine on the NMDA-mediated current was blocked only by the nonselective opioid antagonist naloxone. The κ_1 -selective antagonist Nor-BNI, the μ -selective antagonist cyprodime, and the δ -selective antagonist naltrindole had no effect on bremazocine's inhibition of the NMDA-mediated current. This pattern of antagonism is consistent with the binding selectivities of these agents for the κ_2 binding site (Nock et al., 1988, 1990, 1993; Zukin et al., 1988; Rothman et al., 1992). The concentrations of the antagonists used in this study were well above those previously shown to be effective at blocking opioid agonists (Caudle and Chavkin, 1990; Wagner et al., 1991, 1992, 1993). Also, opioid receptor selectivity of the antagonists breaks down at concentrations above 1 μ M. In addition, the selective antagonists had no effect on the NMDA-mediated current when superfused over the slices alone, indicating that the inhibitory effect of bremazocine and dynor-

phin was not due to antagonism at μ - or δ -opioid receptors. Finally, the approximately 100-fold shift to the right of the bremazocine concentration–response relationship by 100 nM naloxone indicates that naloxone's K_i for this receptor is approximately 7.5 nM. Recent binding studies have reported naloxone's K_i for the κ_2 binding site to be in the range of 7–10.5 nM (Rothman et al., 1992; Nock et al., 1993; Webster et al., 1993). Based on these findings, we concluded that the inhibitory effect of bremazocine and dynorphin was mediated through the κ_2 opioid receptor. In preliminary studies 10 nM bremazocine was found to have no effect on NMDA receptor–mediated synaptic currents. This contrasts with dynorphin's excitatory effects at low concentrations and suggests that bremazocine may be a selective agonist for the κ_2 receptor in this assay. Dynorphin, on the other hand, acts on at least two receptor systems in this assay: an inhibitory opioid (κ_2) receptor and an as yet uncharacterized nonopioid excitatory receptor.

At present, we do not know the mechanism of the κ_2 opioid receptor inhibition of the NMDA receptor–mediated currents. The κ_2 receptors may be directly coupled to the NMDA receptor complex. However, the κ_2 binding sites are located close to the terminals of the dynorphin-containing mossy fibers in the stratum lucidum of the CA3 (McLean et al., 1986; Zukin et al., 1988), whereas the NMDA receptors are located on the distal dendrites (Monaghan and Cotman, 1985) (see Fig. 1). The distance between the κ_2 and the NMDA binding sites suggests that some sort of diffusible, or transportable, second messenger is utilized rather than a direct connection between the receptors. Alternatively, activation of κ_2 receptors may alter the cable properties of the cell, increasing the electrotonic length. Our data suggest that this was not the case because membrane conductance was not altered by dynorphin or bremazocine. Also, non-NMDA currents were not affected in the same manner by these agents as NMDA currents. If the cable properties of the CA3 pyramidal cells were altered by κ_2 receptor activation, we would expect that similar effects would be observed on both NMDA and non-NMDA excitatory amino acid synaptic currents in response to κ_2 opioid receptor activation. However, because the NMDA receptors and their associated ion channels are on the distal dendrites of the CA3 pyramidal cells, we could not be certain of our voltage clamp in that region. Because it is unlikely that the dendrites were actually at +5 mV, it is possible that unobserved changes in membrane properties or voltage-activated ion channels occurred in the distal dendrites, in response to κ_2 agonists, that could be detected only by examining the large NMDA receptor–mediated currents. Future studies will attempt to address this issue more thoroughly.

Data were also presented here suggesting that endogenous dynorphin acts at the κ_2 opioid receptor. Naloxone alone or antibodies to dynorphin (1-17) added to the superfusion buffer increased the NMDA-mediated current, suggesting that an endogenous agonist, presumably dynorphin, was being displaced from the receptor. The selective antagonists NorBNI, cyprodime, and naltrindole as well as antisera to met-enkephalin did not influence the NMDA-mediated synaptic current. These findings support the contention that endogenous dynorphin, or a dynorphin-like peptide, is an endogenous agonist for the κ_2 receptor.

In this study we demonstrated that exogenously applied dynorphin had a biphasic concentration–response relationship (Fig. 2B). At high concentrations exogenous dynorphin inhibited the NMDA receptor–mediated synaptic current, and at low con-

centrations dynorphin enhanced the current. Whether or not endogenous dynorphin acted at the nonopioid site is difficult to determine from the present data. It is possible that dynorphin was released from the mossy fibers directly onto the κ_2 receptors and that the dynorphin did not reach the nonopioid sites, which are presumably on the NMDA receptor complex (see Massardier and Hunt, 1989; Shukla et al., 1992) at some distance from the mossy fibers (Monaghan and Cotman, 1985) (see Fig. 1). This scenario would suggest that the excitatory effect observed in the presence of dynorphin antisera and naloxone was simply the loss of tonic κ_2 receptor inhibition without stimulation at the nonopioid site. Another possibility is that endogenous dynorphin may bind to both sites, with its concentration being high enough for the opioid effect to overwhelm the nonopioid effect. In essence, endogenous dynorphin's concentration was on the inhibitory side of the concentration–response relationship (Fig. 2B). Thus, the excitatory nonopioid effect predominated when dynorphin's concentration was lowered by the antisera or when the opioid receptors were blocked by naloxone. This second hypothesis seems less likely than the first because the excitatory nonopioid effect was clearly observed at low concentrations of exogenously applied dynorphin. If endogenous dynorphin was present at both receptors in sufficient concentration for the opioid effect to predominate, as occurred when 5 μ M dynorphin was applied exogenously (Fig. 2B), the excitatory nonopioid effect could not be observed without first blocking the opioid receptors. Therefore, the most parsimonious explanation for our data was that endogenous dynorphin, or a dynorphin-like peptide, was released by the mossy fibers onto adjacent κ_2 opioid receptors, resulting in tonic inhibition of NMDA receptors while little endogenous opioid reached the nonopioid site. We did not stimulate the mossy fibers in an attempt to release greater amounts of endogenous dynorphin to determine if endogenous dynorphin acted at the nonopioid site. Until we understand what the nonopioid site is, and have a competitive antagonist for that site, attempts to detect endogenous dynorphin's effects on this receptor will be difficult. This task may be especially difficult in the presence of the κ_2 opioid receptor's inhibitory actions.

The results of this study and of previous studies with dynorphin indicate that the role of endogenous dynorphin in the CNS is extremely complex. The results presented here indicate that endogenous dynorphin can bind to κ_2 receptors to inhibit the current that flows through the NMDA receptor complex. This action provides a mechanism for mossy fiber regulation of a synapse that the fibers do not contact directly, the Schaffer collateral/commissural fiber CA3 pyramidal cell synapse. By releasing dynorphin on to κ_2 receptors in the stratum lucidum, the mossy fibers inhibit NMDA receptors in the distal dendrites. We can only speculate as to the importance of this action of endogenous dynorphin. However, it presumably would effect the oscillatory nature of the CA3 pyramidal cells by dampening the recurrent circuitry. In other studies, we found that endogenous dynorphin-like peptides also bind to κ_1 opioid receptors in the guinea pig dentate gyrus (Wagner et al., 1991) and that the κ_1 receptors inhibit excitatory amino acid release from the perforant path (Wagner et al., 1992, 1993). This finding suggests that endogenous dynorphin not only regulates NMDA receptors in the CA3, but that it also regulates excitatory input to the cells that contain the dynorphin: the dentate granule cells. Thus, through the two κ -opioid receptors dynorphin provides a mechanism for dentate granule cells to reduce the conduction of information through the hippocampus.

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