Dynorphin Block of N-Methyl-D-Aspartate Channels Increases with the Peptide Length

LI CHEN and LI-YEN MAE HUANG
Marine Biomedical Institute, University of Texas Medical Branch, Galveston, Texas

ABSTRACT
We examined the non-opioid actions of various forms of dynorphin A (DynA) on N-methyl-D-aspartate (NMDA) receptor channels in isolated rat trigeminal neurons using the whole-cell patch recording technique. All the dynorphins tested blocked NMDA-activated currents. The blocking actions were voltage-independent. The IC_{50} was 0.26 μM for DynA(1-32), 6.6 μM for DynA(1-17), 7.4 μM for DynA(1-13), 42.0 μM for DynA(1-10), DynA(1-8) had no detectable blocking action on NMDA responses. Thus, the IC_{50}s of dynorphins for NMDA receptors increased 160-fold as the length of the peptides decreased from 32 to 10 amino acids. Amidation of dynorphins dramatically reduced their IC_{50}s and eliminated the large difference in the IC_{50}s of various lengths of dynorphins. The reduction in the IC_{50}s of dynorphin amides could not be explained by the resistance of the peptides to enzymatic degradation. Our observations suggest that peptide processing affects dynorphin blocking actions on NMDA responses. The positively charged residues, lengths of the peptides and amidation may contribute to their affinities for NMDA receptors.

The spinal trigeminal nucleus caudalis (the medullary dorsal horn) is the major center involved in processing nociceptive and temperature information. Cells in this region receive inputs fromafferent fibers innervating the face and oral cavity. At the same time, they are subjected to inhibitory controls of the descending pathways and local circuits. Projection neurons then integrate the information and relay it to the thalamus and somatosensory cortex. Glutamate and aspartate are abundantly expressed in dorsal horn neurons (Magnusson, et al., 1986). These amino acids activate both NMDA and non-NMDA receptors and serve as the major excitatory transmitters mediating the synaptic transmission in the nociceptive pathway (Gu and Huang, 1991; Gu and Huang, 1994; Salt and Hill, 1982; Zhang, et al., 1996).

Dynorphins and the mRNA encoding the dynorphin gene, preprodynorphin, are found in the superficial dorsal horns of the spinal cord and of the caudal medulla (Cruz and Basbaum, 1985; Nishimori, et al., 1988; Ruda, et al., 1988). In response to high frequency stimulation of afferent fibers, DynA is released in the laminae I and V-VI of the dorsal horns (Hutchison, et al., 1990). The actions of dynorphins are mostly inhibitory. They reduce the firing frequency of dorsal horn cells and lengthen the latency of tail flick reflexes evoked by noxious electrical, mechanical and thermal stimuli (Caudle and Isaac, 1988; Hope et al., 1990; Knox and Dickenson, 1987; Millan, 1989; Yaksh, 1993). In addition to their inhibitory effects, dynorphins sometimes exert excitatory actions on dorsal horn cells. For instance, dynorphins enhance the glutamate-evoked firing of spinthalamic tract neurons (Willcockson et al., 1986), and enlarge the receptor field of C-fiber or mechanical stimulation (Hylden et al., 1991; Knox and Dickenson, 1987).

Not all the dynorphin actions involve κ-opioid receptors; many are insensitive to high doses of the opioid receptor antagonist, naloxone; some can be mimicked by non-opioid peptide, Des-Tyr DynA (Faden, 1990; Hooke, et al., 1995; Hylden et al., 1991; Knox and Dickenson, 1987; Vanderaa, et al., 1996; Willcockson, et al., 1986). We recently found that dynorphins reduce NMDA-activated currents (I_{NMDA}) in trigeminal neurons (Chen et al., 1995a, 1995b). The actions cannot be reversed by naloxone or by the κ-opioid receptor antagonist, norBNI. In addition, dynorphins reduce the opening probability of single NMDA channels in cell-free patches. These observations have led us to suggest that dynorphins interact directly with NMDA receptors (Chen et al., 1995a, 1995b). We also explored the site of action for dynorphin. Since dynorphin (1-17) changes neither the EC_{50} of NMDA nor the potentiating effect of glycine, the dynorphin does not appear to interact with the agonist recognition sites on NMDA receptors. The binding site for dynorphin is distinct from the sites for H^{+}, Zn^{2+} or Mg^{2+} because the blocking action of dynorphin (1-17) remains unchanged in different

ABBREVIATIONS: DynA, dynorphin A; NMDA, N-methyl-D-aspartate; DTT, diethiothreitol.
pH or Zn\(^{2+}\) solutions and the effect of dynorphin is voltage-independent. Because the IC\(_{50}\) of dynorphin for NMDA receptors is lowered substantially when the disulfide bonds in NMDA receptors are reduced by DTT, we suggest that dynorphin may interact with a site conformationally linked with the redox site(s) on NMDA receptors (Chen, et al., 1995a).

As with many neuropeptides, dynorphins are made from a large precursor molecule, i.e., prodynorphin. In the brain and spinal cord, prohormone convertases cleave prodynorphin at the dibasic residues to generate DynA(1-32), A(1-17) and DynB(1-29) (Cone et al., 1983; Fricker and Devi, 1995; Sanders and Weber, 1987). Dynorphin converting enzyme then processes DynA(1-17) at the single Arg residue to produce DynA(1-9) and DynA(1-8) (Devi et al., 1991). DynA(1-9) is further converted to DynA(1-8) by carboxypeptidase E in the secretory granules before releasing from cells. Because the processing enzymes are differentially expressed in the various brain regions, different lengths of dynorphins are produced in different levels in these locations (Dickerson and Noel, 1991). Although posttranslational processing has been recognized as an important regulatory step in dynorphin biosynthesis, the physiological consequence of this processing has not been fully explored. We therefore studied the actions of various forms of DynA on NMDA responses in trigeminal neurons. We found that different dynorphins exert similar nonopioid blocking actions on NMDA-activated currents. However, their apparent affinities, i.e., IC\(_{50}\)s, for NMDA receptors differ by as much as 200-fold. Positive charges on the dynorphin molecules and conformation of the peptides appear to be important in determining the affinities of these peptides.

### Materials and Methods

Dissociated trigeminal neurons were isolated from 10- to 15-day-old Long Evans rats using the method described (Gu and Huang, 1991; Huang, 1989). In brief, the lower medulla was removed and put in an ice-cold, oxygenated dissecting solution. The solution consisted of (mM) NaCl (120), KCl (10), CaCl\(_2\) (1), MgCl\(_2\) (6), glucose (10) and PIPES (10) (pH = 7.15) (osmolarity = 305-315 mosm). The tissue was cut into 300 \(\mu\)m thick horizontal slices with a vibratome slicer and incubated in the dissecting solution at 34.5°C for 30 min. The slices were then put in a dissecting solution that contained 2.5 mg/ml papain (Sigma P3250). After 40- to 60-min incubation period, the tissue was washed with enzyme-free dissecting solution and stored at room temperature. Before an experiment, the spinal trigeminal nuclei in the caudal medulla were isolated from a tissue slice with a scalpel. Neurons were isolated by triturating the tissue with a series of fire-polished Pasteur pipettes.

Whole-cell 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). Experiments were performed in 0 Mg\(^{2+}\) and 2 \(\mu\)M glycine. Because the treatment of the AMPA receptor antagonist, CNQX, did not change our results, most experiments were conducted in the absence of a non-NMDA receptor antagonist. The internal solution contained (mM) Cs methanesulfonate (125), CsCl (15), glucose (10), BAPTA (10), CaCl\(_2\) (1) and HEPES (10) (pH = 7.2). Mg-ATP (5 mM), leupeptin (400 \(\mu\)M) (Sigma Chemical Co., St. Louis, MO) and GTP (200 \(\mu\)M) were added to the internal solution to prevent the rundown. All chemicals are ultrapure grade. Dynorphins (Peninsula Lab, Belmont, CA) were dissolved in the external solution immediately before use.

NMDA was delivered to the recorded cell using the fast perfusion technique (Gu and Huang, 1991). The solution exchange was accomplished in 10 msec. Each NMDA application lasted for 1.5 sec. To avoid excessive desensitization or rundown of the I\(_{\text{NMDA}}\) caused by repeated NMDA applications, we washed NMDA out completely after each application and waited 2 to 3 min before another NMDA application. With this precaution, I\(_{\text{NMDA}}\) decayed rather slowly during the course of experiments. To take into account the current decay, two NMDA responses were recorded in a dynorphin solution and the responses were compared with the NMDA responses taken immediately before the dynorphin application.

The currents were sampled at 200 \(\mu\)sec and signals were filtered at 2 kHz. The \(\chi^2\) method was used to analyze the dose-inhibition curves. The average data values are expressed as mean \(\pm\) S.E.

A Beckman reverse-phase column (0.46 \(\times\) 25 cm, ODS, C18) was used for the HPLC analysis. The column was preequilibrated with 1% aqueous trifluoroacetic acid. The peptides were eluted with a linear 0 to 60% acetonitrile gradient. The eluted material was monitored at 230 nm with a Beckman Gold System 406.

### Results

The IC\(_{50}\)s of dynorphins increase as the peptides become shorter. Figure 1 shows the effect of various dynorphins on NMDA responses in trigeminal neurons. At the holding potential of -70 mV, NMDA (100 \(\mu\)M) activated large inward currents (I\(_{\text{NMDA}}\)). After incubating cells in dynorphin-containing external solution for 2 min, NMDA responses were tested again. Dynorphins reduced the I\(_{\text{NMDA}}\) (fig. 1A). The block was rapid and could be readily reversed.
when dynorphins were washed out (Chen et al., 1995a, 1995b) (data not shown). Opioid receptors were not involved because the inhibition could not be reversed by naloxone or by nor-BNI (data not shown). The dynorphin blocking effect was observed in all of the cells that responded to NMDA.

When the blocking actions of the three naturally occurring dynorphins, i.e., DynA(1-32), DynA(1-17) and DynA(1-8), were studied, we found that the potency of the dynorphins depended on their lengths (fig. 1A). The longer the dynorphin, the more potent was the block. The dose-inhibition curves of these dynorphins were similar in shape (Hill coefficient = 1) (fig. 1B). But, the IC50 of the block changed dramatically with the length of the molecules. The IC50 was 42.0 ± 6.0 (n = 5) μM for DynA(1-10), 7.4 ± 1.9 μM (n = 6) DynA(1-13), 6.6 ± 1.1 μM (n = 5) for DynA(1-17) and 0.26 ± 0.03 (n = 7) μM for DynA(1-32). Thus, the IC50 decreased or the apparent affinities increased 160-fold as the length of the dynorphins increased from 10 to 32 amino acids. DynA(1-8), even at 100 μM, had no detectable blocking action on NMDA responses (fig. 1A). The IC50 of DynA(1-13) and of DynA(1-17) obtained here (fig. 1B) are much larger than those reported in our previous report (Chen et al., 1995b). The reason is that amidated forms of DynA(1-13) and of DynA(1-17) were used before. The difference between the carboxyl and amidated forms of dynorphins will be discussed later. The effect of DynB (1-13) on NMDA currents was also tested; DynB blocked NMDA current with an IC50 of 38.8 ± 6.8 μM (n = 3).

**The blocking actions of various dynorphins are voltage-independent.** We found that the block of NMDA responses by DynA(1-32) was voltage independent (Chen et al., 1995b). To determine if shorter dynorphins had the similar blocking property, we examined the action of DynA(1-10) at different membrane potentials. The current-voltage relationship of the peptide was given in figure 2A. DynA(1-10) blocked INMDA to a similar extent as the membrane potentials changed from -80 to +60 mV (fig. 2). Thus, despite a 160-fold difference in the apparent affinities, DynA(1-32) and DynA(1-10) both exerted voltage-independent actions on NMDA receptors.

Amidation of dynorphins increases the apparent affinity of the peptides for NMDA-receptor channels. To find out if the negative charge at the C-terminus of the dynorphins was important in determining their affinities for NMDA-receptor channels, we compared the IC50 of dynorphins and dynorphin amides. The IC50 of DynA(1-17) amide was 1.7 ± 0.3 μM (n = 6), which was 4-fold lower than that of DynA(1-17) (fig. 3). The difference between the IC50 of DynA(1-10) amide and of DynA(1-10) was even larger (fig. 3). The IC50 of DynA(1-10) amide was 1.7 ± 0.2 μM (n = 5); the IC50 of DynA(1-10) was 42.0 ± 6.0 μM (n = 5). Amidation resulted in a ~25-fold reduction in the IC50. Furthermore, in sharp contrast to the large differences in the IC50 of the unamidated dynorphins, the IC50s of various dynorphin amides were very similar. Thus, the negative charge at the C-terminus of the dynorphins has a profound effect on their affinities for NMDA receptor channels.

**Resistance to enzymatic degradation cannot explain the large reduction in the IC50 of the dynorphin amides.** It is often suggested that α-amidation renders a peptide less susceptible to exopeptidase degradation (McKnight et al., 1983). To find out if this characteristic contributed to the impressive reduction in the IC50 of the amidated dynorphins, we carried out two types of experiments. First, we determined the degradation products in our native dynorphin or dynorphin amide solutions using the HPLC. No extra degradation products were found in the HPLC profile of DynA(1-10) even after the peptide had been incubated in the external solution for an extended period (>60 min) (fig. 4). Second, we examined the IC50 of DynA(1-10) in an external solution that contained a cocktail of peptidase inhibitors (fig. 5). The cocktail, which consisted of 20 μM bestatin, 10 μM captopril, 0.3 μM thiorphan and 2 mM Leu-leucine, is known...
to block a broad spectrum of peptidases (McKnight et al., 1983). In the presence of the peptidase inhibitors, the IC\textsubscript{50} of DynA(1-10) was 46.2 ± 6.7 \(\mu\)M (\(n = 4\)); the IC\textsubscript{50} obtained in the absence of inhibitors was 42.0 ± 6.0 \(\mu\)M (\(n = 5\)). The difference was not statistically significant.

DynA(1-10) and DynA(1-10) amide compete for the same binding site on NMDA-receptor channels. We also determined whether DynA(1-10) and DynA(1-10) amide acted on the same receptor site. The IC\textsubscript{50} of DynA(1-10) amide was obtained in the presence of 40 \(\mu\)M DynA(1-10). If the two peptides competed for the same binding site, the IC\textsubscript{50} of DynA(1-10) amide is expected to increase. This was indeed the case. The IC\textsubscript{50} of DynA(1-10) amide changed from 1.7 ± 0.2 \(\mu\)M to 3.6 ± 0.3 \(\mu\)M (\(n = 5\)) when DynA(1-10) was added to the external solution (fig. 6). We further determined if the change in the IC\textsubscript{50} could be quantitatively accounted for by a single site model, i.e. the binding of one molecule excludes the binding of the second molecule. According to the model, the IC\textsubscript{50} of DynA(1-10) amide in the presence of DynA(1-10), i.e., IC\textsubscript{50-amide}, is

\[
\text{IC}_{50}^{\text{amide}} = \frac{1 + [\text{DynA10}]}{[\text{DynA10}] / \text{IC}_{50}^{\text{DynA10}}}
\]

where IC\textsubscript{50-amide} is the IC\textsubscript{50} of DynA(1-10) amide without DynA(1-10), [DynA10] is the concentration and IC\textsubscript{50-DynA10} is the IC\textsubscript{50} of DynA(1-10). Using IC\textsubscript{50-DynA10} = 42 \(\mu\)M, [DynA10] = 40 \(\mu\)M, the calculated IC\textsubscript{50-amide} is 3.3 \(\mu\)M, in good agreement with our experimental results.

Discussion

We confirmed and extended our previous observation that dynorphins directly interact with NMDA receptors (Chen et al., 1995a, 1995b). The apparent affinities of dynorphins depend on the length of peptides. In addition, we found that \(\alpha\)-amidation not only increases the dynorphin apparent affinities for NMDA receptors, but also abolishes the large differences in the affinities of DynA(1-17), DynA(1-13) and DynA(1-10) (fig. 3). The lack of degradation products in the HPLC profile (fig. 4) and the insensitivity of the IC\textsubscript{50} of DynA(1-10) to peptidase inhibitors (fig. 5) suggest that the high affinities of dynorphin amides do not result from their resistance to enzymatic degradation. Rather, our results are consistent with the idea that amidation eliminates the negative charge at the C-terminus of the dynorphins, rendering them better able to interact with NMDA receptors.

The charge group at the C-terminus is not the only determinant for the binding of dynorphins. The positively charged amino acids, Arg and Lys might facilitate the interaction with NMDA receptors. The amino acid sequences and charge distributions of dynorphins are given in figure 7. Among the dynorphins tested, DynA(1-32), with three Arg and two Lys residues extended beyond Gln\textsuperscript{17}, has the highest affinity for NMDA receptors despite its terminal COO\textsuperscript{−} group (fig. 1). DynA(1-17) and DynA(1-13), with the same number of Arg and Lys groups, have the same IC\textsubscript{50}. Without the two Lys groups toward the C-terminal end, the apparent affinity of DynA(1-10) is further reduced by ~7 fold (fig. 3). Eliminating one more Arg causes DynA(1-8) to lose its ability to bind NMDA receptors. Without any Arg or Lys, Leu-enkephalin and Leu-enkephalin amide have almost no affinity for NMDA receptors (fig. 7). It is conceivable, however, that the conformation of dynorphins derived from the positive charges, rather than the positive charges themselves, is the major determinant for the interaction between dynorphins and NMDA receptors. We therefore conclude that the amidation, the positively charged groups and the lengths of dynorphins, which contribute to the three-dimensional conformation of the molecules, are important in determining their binding affinities for NMDA receptors.

Because the dynorphin block of NMDA receptor channels occurs at high opioid concentrations (>100 nM), it may argue that this nonopioid effect would not be physiologically impor-
tant. This argument, however, ignores the anatomical relationship between opioid peptide-containing terminals and opioid receptors. Mu (μ)- and κ-opioid receptors primarily localize on somatodendritic membrane; many are found at extrasynaptic sites, away from opioid-containing terminals (Arvidsson et al., 1995a, 1995b; Svingos et al., 1996). These observations suggest that opioids, once released, will travel considerable distances before reaching opioid receptors. It is therefore not surprising to find opioid receptors displaying high affinity for their ligands. In contrast, glutamate receptors are found to associate with synaptic terminals (Martin et al., 1993; Siegel et al., 1994). Opioids and substance P are found to colocalize in nerve terminals (Tashiro et al., 1987; Weihe et al., 1986), a majority of which may be glutamate-positive (Battaglia and Rustioni, 1988). Using known dynorphin concentration in secretory vesicles, we estimated that the dynorphins at postsynaptic NMDA receptors would exceed micromolars (Chen et al., 1995a), a concentration range that could affect NMDA responses.

Bioactive opioid peptides are derived from large precursors (i.e., POMC, proenkephalin and prodynorphin) that undergo posttranslational processing. One distinct feature of peptide processing is tissue specificity (Cone et al., 1983; Fricker and Devi, 1995; Sonders and Weber, 1987). Expressing in different levels, processing enzymes cleave prodynorphin into distinct sets of dynorphin molecules in different tissues. The concentrations of various dynorphins, therefore, vary markedly among brain regions. In the caudate, DynA(1-8)/DynA(1-17)≈2 in the hypothalamus and ≈1 in the hippocampus (Cone et al., 1983; Sonders and Weber, 1987). In the spinal cord, the ratio is reversed, that is, DynA(1-7) is 1.25 to 2.8 times more abundant than DynA(1-8) (Cone et al., 1983; Sonders and Weber, 1987). A physiological consequence of the varying dynorphin concentration ratios can be deduced from our results. Because DynA(1-17) has a much higher affinity for NMDA receptors than DynA(1-8), the non-opioid blocking actions of dynorphins would be prominent in the spinal cord, but less so in the caudate. Nevertheless, the nonopioid effect may not be negligible in other regions of the brain because a substantial amount of DynA(1-17) is produced in those areas (Cone et al., 1983; Sonders and Weber, 1987). Thus, because different dynorphins have different affinities for NMDA receptors (fig. 1) and possess different metabolic stability (Corbett et al., 1982), the posttranslational processing of dynorphins is a likely mechanism used for regulating the function of the peptides.

In addition to the tissue variability, processing of opioid peptide precursors can be altered temporally (Dickerson and Noël, 1991). Although the effect of tissue injury on opioid processing has yet to be examined, plastic changes in dynorphin distribution in the spinal and trigeminal dorsal horns following inflammation or arthritis have been observed. Compared with other opioid peptides, dynorphins are particularly sensitive to tissue injury. Chronic arthritic inflammation raises the prodynorphin and the level of dynorphin synthesis up to 2- to 4-fold in the dorsal horns, whereas proenkephalin increases only moderately (~50%) (Kajander et al., 1990; Millan, 1993; Ruda et al., 1988; Weihe et al., 1989). Under normal physiological conditions, the dynorphin concentrations probably are not high enough to saturate the non-opioid effect of dynorphins (fig. 1) (Chen et al., 1995b). An increase in dynorphin expression after tissue injury would dramatically increase its blocking action on NMDA responses. Furthermore, the mRNAs encoding the prohormone convertases I and II are found to increase rapidly after electroconvulsive treatment in the hippocampus (Bhat et al., 1993). Because tissue injury is always accompanied with intensive electrical activities, the relative concentration ratios of dynorphins are likely to change. This would alter the nonopioid actions of dynorphins, further contributing to the plasticity of dynorphin functions.

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Send reprint requests to: Dr. Li-Yen Mae Huang, Marine Biomedical Institute, University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77555-1069.