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N-METHYL-D-ASPARTATE RECEPTORS AND LARGE CONDUCTANCE CALCIUM-SENSITIVE POTASSIUM CHANNELS INHIBIT THE RELEASE OF OPIOID PEPTIDES THAT INDUCE μ-OPIOID RECEPTOR INTERNALIZATION IN THE RAT SPINAL CORD

B. SONG AND J. C. G. MARVIZÓN*

Center for Neurovisceral Sciences and Women's Health and CURE: Digestive Diseases Research Center, Division of Digestive Diseases, Department of Medicine, David Geffen School of Medicine at UCLA and Veteran Affairs Greater Los Angeles Healthcare System, Building 115, 11301 Wilshire Boulevard, Los Angeles, CA 90073, USA

Abstract—Endogenous opioids in the spinal cord play an important role in nociception, but the mechanisms that control their release are poorly understood. To simultaneously detect all opioids able to activate the μ-opioid receptor, we measured μ-opioid receptor internalization in rat spinal cord slices stimulated electrically or chemically to evoke opioid release. Electrical stimulation of the dorsal horn in the presence of peptidase inhibitors produced μ-opioid receptor internalization in half of the μ-opioid receptor neurons. This internalization was rapidly abolished by *N*-methyl-D-aspartate (IC_{50}=2 μM), and *N*-methyl-D-aspartate antagonists prevented this effect. μ-Opioid receptor internalization evoked by high K^+ or veratridine was also inhibited by *N*-methyl-D-aspartate receptor activation. *N*-methyl-D-aspartate did not affect μ-opioid receptor internalization induced by exogenous endomorphins, confirming that the effect of *N*-methyl-D-aspartate was on opioid release. We hypothesized that this inhibition was mediated by large conductance Ca^{2+}-sensitive K^+ channels BK(Ca^{2+}). Indeed, inhibition by *N*-methyl-D-aspartate was prevented by tetraethylammonium and by the selective BK(Ca^{2+}) blockers paxilline, penitrem A and verruculogen. Paxilline did not increase μ-opioid receptor internalization in the absence of *N*-methyl-D-aspartate, indicating that it does not produce an increase in opioid release unrelated to the inhibition by *N*-methyl-D-aspartate. The BK(Ca^{2+}) involved appears to be a subtype with slow association kinetics for iberiotoxin, which was effective only with long incubations. The BK(Ca^{2+}) opener NS-1619 also inhibited the evoked μ-opioid receptor internalization, and iberiotoxin prevented this effect. We concluded that Ca^{2+} influx through *N*-methyl-D-aspartate receptors causes the opening of BK(Ca^{2+}) and hyperpolarization in opioid-containing dorsal horn neurons, resulting in the inhibition of opioid release. Since μ-opioid receptors in the dorsal horn mediate analgesia, inhibition of spinal opioid release could contribute to the hyperalgesic actions of spinal *N*-methyl-D-aspartate receptors. **© 2005 Published by Elsevier Ltd on behalf of IBRO.**

Key words: dorsal horn, dynorphin, enkephalin, internalization, mu-opioid receptor, opioid.

*Corresponding author. Tel: +1-310-478-3711x41850; fax: +1-310-312-9289.
E-mail address: marvizon@ucla.edu (J. C. G. Marvizón).

Abbreviations: aCSF, artificial cerebrospinal fluid; ANOVA, analysis of variance; AP-5, DL-2-amino-5-phosphonopentanoic acid; BK(Ca^{2+}), large conductance Ca^{2+}-sensitive K^+ channels; CCK, cholecystokinin; CCK-8, cholecystokinin-8; C.I., confidence interval; CPP, (*RS*)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid; DAMGO, [D-Ala[2],NMe-Phe[4],Gly-ol[5]]enkephalin; DCG-IV, (2*S*,2′*R*,3′R)-2-(2′,3′-dicarboxycyclopropyl)-glycine; DHPG, (*RS*)-3,5-dihydroxyphenylglycine; DPDPE, [2-D-penicillamine, 5-D-penicillamine]-enkephalin; IC_{50}, effective concentration of drug for 50% of the inhibition; K^+-aCSF, aCSF with 5 mM KCl; L-AP4, L-(+)-2-amino-4-phosphonobutyric acid; LY-341495, (2*S*)-2-amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid; mGluR, metabotropic glutamate receptor; MK-801, dizocilpine, (5*R*,10*S*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[a,d]cyclohepten-5,10-imine hydrogen maleate; MOR, μ-opioid receptor; NBQX, 2,3-dioxo-6-nitro-1,2,3,4,-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide; n_H, Hill coefficient; NMDA, *N*-methyl-D-aspartate; NS-1619, 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)-phenyl]-5-(trifluoromethyl)-2*H*-benzimidazol-2-one; SDZ-220-040, (*S*)-α-amino-2′,4′-dichloro-4-hydroxy-5-(phosphonomethyl)-[1,1′-biphenyl]-3-propanoic acid; sucrose-aCSF, artificial cerebrospinal fluid with 5 mM KCl and 215 mM sucrose instead of NaCl; TEA, tetraethylammonium.

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Alkaloid opiates acting on μ-opioid receptors (MORs) are the most powerful analgesics available, but they produce tolerance and addiction. Physiologically, MORs are activated by opioid peptides, and strategies that increase the availability of these opioids by inhibiting their degradation have been shown to produce analgesia (Chou et al., 1984; Fournie-Zaluski et al., 1992; Noble et al., 1992b). Moreover, there is some evidence that this approach produces little tolerance (Noble et al., 1992c) and dependence (Noble et al., 1992a). One way to increase opioid availability would be by targeting neurotransmitter receptors that control opioid release; however, these are largely unknown. One group has reported that Met-enkephalin release in the spinal cord is increased by neuropeptide FF (Ballet et al., 1999; Mauborgne et al., 2001) and inhibited by μ and δ autoreceptors (Bourgoin et al., 1991; Collin et al., 1994; Mauborgne et al., 2001). Other investigators (Przewlocka et al., 1990) found that spinal release of α-neoendorphin was increased by noradrenaline and inhibited by $GABA_A$ receptors. However, the physiological relevance of these effects remains unclear.

Our previous studies (Song and Marvizon, 2003a,b) indicated that internalization of MORs in dorsal horn neurons evoked by high K^+, veratridine or electrical stimulation reflects the release of enkephalins and dynorphins from other dorsal horn interneurons (Todd and Spike, 1993). Studying opioid release is particularly challenging because, whereas post-translational processing of opioid gene products produces many active peptides (Yaksh et al., 1983), the immunoassays commonly used to measure opioid release detect just one of them, and therefore are poor predictors of opioid receptor activation. In contrast, MOR internalization can be used to simultaneously detect the release of all opioid pep-

tides able to activate this receptor (Eckersell et al., 1998; Marvizon et al., 1999; Trafton et al., 2000; Song and Marvizon, 2003a,b; Mills et al., 2004). Although morphine and other alkaloid opiates can activate the MOR without inducing its internalization (Whistler et al., 1999), all physiologically-occurring opioids tested produce MOR internalization (Trafton et al., 2000; Song and Marvizon, 2003a). Further evidence that MOR internalization follows its activation by peptides is that the potency of [D-Ala^2,NMe-Phe^4,Gly-ol^5]-enkephalin (DAMGO) to produce MOR internalization is the same as its potency to increase [γ-^{35}S]GTP binding and to inhibit adenylyl cyclase (Marvizon et al., 1999), and that DAMGO injected intrathecally produced spinal MOR internalization and behavioral analgesia at the same doses (Trafton et al., 2000).

In the present study we used stimulus-evoked MOR internalization in dorsal horn neurons to identify neurotransmitter receptors that modulate spinal opioid release. We found that activation of $GABA_A$, $GABA_B$, δ-opioid, cholecystokinin (CCK) and metabotropic glutamate receptors (mGluRs) does not affect spinal opioid release. However, activation of *N*-methyl-D-aspartate (NMDA) receptors produces a robust inhibition of spinal opioid release by opening large conductance Ca^{2+}-dependent K^+ channels (maxi-K or BK(Ca^{2+})). Because MORs in the dorsal horn mediate analgesia (Yaksh, 1997), this is consistent with the sensitization to pain produced by spinal NMDA receptors (Dingledine et al., 1999; Brauner-Osborne et al., 2000; South et al., 2003).

EXPERIMENTAL PROCEDURES

All animal procedures were approved by the Chancellor's Animal Research Committee at UCLA and conform to NIH guidelines. Efforts were made to minimize the number of animals and their suffering.

Chemicals

Baclofen, 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)-phenyl]-5-(trifluoromethyl)-2*H*-benzimidazol-2-one (NS-1619), endomorphin-2, L-glutamic acid, iberiotoxin, ifenprodil, isoguvacine, ketamine, MK-801, NMDA, paxilline, [2-D-penicillamine, 5-D-penicillamine]-enkephalin (DPDPE), penitrem A, D-serine, tetraethylammonium (TEA), veratridine, and verruculogen were purchased from Sigma-RBI (St. Louis, MO, USA). (2*S*)-2-amino-2-[(1*S*,2*S*)-2-carboxycycloprop-1-yl]-3-(xanth-9-yl) propanoic acid (LY-341495), (*S*)-α-amino-2′,4′-dichloro-4-hydroxy-5-(phosphonomethyl)-[1,1′-biphenyl]-3-propanoic acid (SDZ-220-040), L-(+)-2-amino-4-phosphonobutyric acid (L-AP4), DL-2-amino-5-phosphonopentanoic acid (AP-5), (*RS*)-3-(2-carboxypiperazin-4-yl)-propyl-1-phosphonic acid (CPP), (2*S*,2′*R*,3′*R*)-2-(2′,3′-dicarboxycyclopropyl)-glycine (DCG-IV), 5,7-dichloro-kynurenic acid, (*RS*)-3,5-dihydroxyphenylglycine (DHPG), and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide (NBQX) were purchased from Tocris (Ellisville, MO, USA). Cholecystokinin-8 (CCK-8) was a gift from Dr. Joseph Reeves, Division of Digestive Diseases, UCLA (Los Angeles, CA, USA). Isoflurane was from Halocarbon Laboratories, River Edge, NJ, USA.

Spinal cord slices

Media used for the slices were: artificial cerebrospinal fluid (aCSF), containing (in mM) 124 NaCl, 1.9 KCl, 26 $NaHCO_3$, 1.2 KH_2PO_4, 1.3 $MgSO_4$, 2.4 $CaCl_2$ and 10 glucose; K^+-aCSF, containing 5 mM of KCl, and sucrose-aCSF, the same as K^+-aCSF except that NaCl was iso-osmotically replaced with sucrose (215 mM, as assessed with an osmometer). Coronal spinal cord slices were prepared as previously described (Lao et al., 2003; Song and Marvizon, 2003a,b). Briefly, the spinal cord was extracted from 3 to 4 weeks old male Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) under isoflurane anesthesia. Coronal slices (400 μm) without roots were cut with a Vibratome (Technical Products International, St. Louis, MO, USA) in ice-cold sucrose-aCSF. Up to six slices from each animal were cut sequentially in the L1–L4 region.

Slice stimulation

Slices were stimulated electrically at the dorsal horn as described (Song and Marvizon, 2003b). Briefly, a coronal slice was held vertically in a superfusion chamber with stainless steel insect pins and a stimulating electrode was placed with its poles on either side of one dorsal horn (i.e. current flow is oriented rostro-caudally). The shape and size of the stimulating electrode was such that it completely covered one of the dorsal horns ("hook" shape with 1 mm diameter, made from two 0.25 mm platinum/iridium parallel wires separated 1 mm; purchased from Frederick Haer & Co., Bowdoinham, ME, USA). The non-stimulated side of the slice was marked with a round hole in the ventral horn. Electrical stimulation typically consisted of 1000 square pulses (30 V, 0.4 ms) delivered at 500 Hz; frequencies of 10 Hz, 30 Hz or 100 Hz were used in some experiments. Peptidase inhibitors (10 μM actinonin, captopril and phosphoramidon, with 6 μM dithiothreitol) and other drugs were superfused starting 5 min before and ending 5 min after the stimulation. NMDA and D-serine were superfused for 2.5 min, ending with the stimulation. Other slices were stimulated chemically by incubating them with 50 mM KCl for 2 min or with 20 μM veratridine for 1 or 2 min (Song and Marvizon, 2003a). Slices were fixed 5 min after the end of the stimulation. Treatments were randomized between slices, and no more than two slices from the same animal received the same treatment.

Immunohistochemistry

Histological sections of 25 μm were cut from the slices and labeled as previously described (Marvizon et al., 1999; Song and Marvizon, 2003a,b). To label MORs we used a rabbit antiserum (1:7000 dilution) raised against amino acids 384–398 of the cloned rat MOR-1 (DiaSorin, Stillwater, MN, USA, catalog no. 24216). This antiserum has been characterized (Arvidsson et al., 1995) and shown to label dorsal horn neurons (Spike et al., 2002). Pre-absorption of the MOR antibody with its immunizing peptide (10 μg/ml) abolished the staining. The secondary antibody was Alexa-488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA), used at 1:2000 dilution for 2 h at room temperature. Sections were mounted in Prolong (Molecular Probes).

Confocal microscopy

Confocal images were acquired at UCLA's Carol Moss Spivak Cell Imaging Facility with a Leica TCS-SP confocal microscope. Low magnification images were obtained with a 20× objective and consist of one optical section 2.53 μm thick (full width half maximum), or two optical sections 2.53 μm thick separated 2.48 μm. High magnification images were obtained with a 100× objective and consist of two to three optical sections 0.62 μm thick, separated 0.57 μm. The pinhole was 1.0 Airy units. Optical sections were averaged four times to reduce noise. Images were processed using Adobe Photoshop 5.5. The "curves" feature of the program was used to adjust the contrast. Images were acquired at a digital size of 1024×1024 pixels and were later cropped to the relevant part of the field.

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