Published in final edited form as:

Neuropharmacology. 2008 May ; 54(6): 944–953. doi:10.1016/j.neuropharm.2008.02.002.

Inhibition of opioid release in the rat spinal cord by α_{2C} adrenergic receptors

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Abstract

Neurotransmitter receptors that control the release of opioid peptides in the spinal cord may play an important role in pain modulation. Norepinephrine, released by a descending pathway originating in the brainstem, is a powerful inducer of analgesia in the spinal cord. Adrenergic α_{2C} receptors are present in opioid-containing terminals in the dorsal horn, where they could modulate opioid release. The goal of this study was to investigate this possibility. Opioid release was evoked from rat spinal cord slices by incubating them with the sodium channel opener veratridine in the presence of peptidase inhibitors (actinonin, captopril and thiorphan), and was measured in situ through the internalization of μ -opioid receptors in dorsal horn neurons. Veratridine produced internalization in 70% of these neurons. The α_2 receptor agonists clonidine, guanfacine, medetomidine and UK-14304 inhibited the evoked μ -opioid receptor internalization with IC_{50} s of 1.7 μ M, 248 nM, 0.3 nM and 22 nM, respectively. However, inhibition by medetomidine was only partial, and inhibition by UK-14304 reversed itself at concentrations higher than 50 nM. None of these agonists inhibited μ -opioid receptor internalization produced by endomorphin-2, showing that they inhibited opioid release and not the internalization itself. The inhibition produced by clonidine, guanfacine or UK-14304 was completely reversed by the selective α_{2C} antagonist JP-1203. In contrast, inhibition by guanfacine was not prevented by the α_{2A} antagonist BRL-44408. These results show that α_{2C} receptors inhibit the release of opioids in the dorsal horn. This action may serve to shut down the opioid system when the adrenergic system is active.

Keywords

Clonidine; dorsal horn; dynorphin; enkephalin; guanfacine; internalization; JP-1302; medetomidine; μ -opioid receptor; norepinephrine; opioid; UK-14304

1. Introduction

Norepinephrine and opioid peptides (henceforth, “opioids”) are among the most powerful inducers of analgesia in the spinal cord. The analgesic effect of opioids (Chen and Pan, 2006; Russell et al., 1987) is mediated by μ -opioid receptors (MORs) and δ -opioid receptors (Budai and Fields, 1998; Chen et al., 2007b; Takemori and Portoghese, 1993), while that of

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norepinephrine is largely mediated by adrenergic α_2 receptors (Pertovaara, 2006; Yaksh, 1985). Physiologically, norepinephrine-induced analgesia is driven by a descending pain inhibitory pathway originating in three adrenergic nuclei: A5, A7 and nucleus coeruleus (A6) (Pertovaara, 2006). Spinal cord opioids derive from the pre-proenkephalin and pre-prodynorphin genes, which are expressed in different interneurons (Cruz and Basbaum, 1985). At present, it is not clear to what extent spinal opioid release is driven by a brainstem descending pathway (Jensen and Yaksh, 1984; Morgan et al., 1991; Zorman et al., 1982) or by local dorsal horn circuits (Cesselin et al., 1989; Le Bars et al., 1987a; Le Bars et al., 1987b; Song and Marvizon, 2003b). Synergism between the analgesic effects of opioid and α_2 adrenergic receptors (Bohn et al., 2000; Stone et al., 1997a; Wei et al., 1996) suggests that there are separate noradrenergic and opioidergic pain inhibitory pathways. However, the mechanisms by which these two pathways interact with each other remain unclear.

Of the three subtypes of α_2 receptors, α_{2A} receptors are present in the primary afferent terminals and α_{2C} receptors are located in the presynaptic terminals of excitatory interneurons (Marvizon et al., 2007; Olave and Maxwell, 2002, 2003a, b, 2004; Stone et al., 1998), whereas expression of α_{2B} receptors in the dorsal horn is quite limited (Shi et al., 1999). Noradrenergic analgesia is thought to be mediated primarily by α_{2A} receptors, which inhibit glutamate release from primary afferent terminals. Additionally, α_2 receptors hyperpolarize dorsal horn neurons (North and Yoshimura, 1984). Noradrenergic analgesia may also involve α_{2C} receptors (Fairbanks et al., 2002), which could block excitatory input onto projection neurons (Olave and Maxwell, 2003a, b, 2004).

The fact that presynaptic terminals that have α_{2C} receptors also contain opioids (Marvizon et al., 2007; Olave and Maxwell, 2002; Stone et al., 1998) raises the possibility that α_{2C} receptors control opioid release. They probably inhibit opioid release, because α_2 receptors couple to α_i G-proteins and inactivate Ca^{2+} channels (Summers and McMartin, 1993). We investigated this possibility by studying the effect of α_2 agonists and antagonists on opioid release evoked in rat spinal cord slices by veratridine (Przewlocka et al., 1990; Song and Marvizon, 2003a; Song and Marvizon, 2005; Uzumaki et al., 1984). Opioid release was measured in situ by the internalization of MORs, an approach validated in the spinal cord (Chen et al., 2007b; Song and Marvizon, 2003a; 2003b; Song and Marvizon, 2005; Trafton et al., 2000) and the brain (Eckersell et al., 1998; Mills et al., 2004; Sinchak and Micevych, 2001). MOR internalization correlates well with opioid-induced analgesia and other measures of MOR activation (Chen et al., 2007b; Marvizon et al., 1999; Trafton et al., 2000). Parts of this study have been published as an abstract (Chen et al., 2007a).

2. Methods

Animal procedures were approved by the Institutional Animal Care and Use Committee of the Veteran Affairs Greater Los Angeles Healthcare System, and conform to NIH guidelines. Efforts were made to minimize the number of animals and their suffering.

2.1. Spinal cord slices

Coronal spinal cord slices were prepared as previously described (Marvizon et al., 1999; Song and Marvizon, 2003a; 2003b; Song and Marvizon, 2005). Media used for the slices were: 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). The spinal cord was extracted from 3–4 weeks old male Sprague-Dawley (Harlan, Indianapolis, IND) rats under isoflurane anesthesia. Coronal slices (400 μm) were cut with a Vibratome (Technical Products International, St. Louis, MO) in ice-cold sucrose-aCSF. Up to six slices from each animal were cut sequentially in the L1–L4 region.

2.2. Slice stimulation

To evoke opioid release, slices were incubated with 20 μ M veratridine for 2 min at 35 °C, as previously described (Song and Marvizon, 2003a; 2005). Veratridine increases Na⁺ fluxes through voltage-dependent Na⁺ channels (Satoh and Nakazato, 1991), and evokes the release of enkephalin (Uzumaki et al., 1984) and dynorphin (Przewlocka et al., 1990) from the spinal cord. The evoked MOR internalization was maximal with veratridine concentrations of 10 μ M–30 μ M and incubation times of 0.5–2 min. For the incubation, the slices were placed on a nylon net glued to a plastic ring inserted halfway down a plastic tube containing 5 ml aCSF. The aCSF was superficially gassed with a flow of 95% O₂/5% CO₂ passing through a needle inserted through the cap of the tube. To change solutions, the ring and net with the slice was transferred to a different tube. We previously found that MOR internalization induced by released opioids can only be observed if the opioids are protected from peptidase degradation (Song and Marvizon, 2003a). Accordingly, the slices were incubated with peptidase inhibitors (10 μ M actinonin, captopril and thiorphan) starting 5 min before and ending 10 min after the addition of veratridine. Drugs tested in the experiments were added to the slices at the same time as the peptidase inhibitors, except when stated otherwise. After the 2 min incubation with veratridine, the slices were incubated for 10 min more at 35 °C with peptidase inhibitors and other drugs to allow the completion of MOR internalization (Marvizon et al., 1999). At the end of the incubation the slices were fixed by immersion in ice-cold fixative (4% paraformaldehyde, 0.18 % picric acid in 0.1 M sodium phosphate buffer).

2.3. Immunohistochemistry

Histological sections of 25 μ m were cut from the slices and labeled as previously described (Marvizon et al., 1999; Song and Marvizon, 2003a; Song and Marvizon, 2003b, 2005). To label MORs we used a rabbit antiserum (1:7000 dilution) raised against amino acids 384–398 of the cloned rat MOR-1 (ImmunoStar, Hudson, WI, 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 μ g/ml) abolished the staining. The secondary antibody was Alexa-488 goat anti-rabbit IgG (Molecular Probes, Eugene, OR), used at 1:2000 dilution for 2 hr at room temperature. Sections were mounted in Prolong Gold with DAPI (Molecular Probes).

2.4. Quantification of MOR internalization

MOR internalization was quantified as previously described (Marvizon et al., 1999; Song and Marvizon, 2003a; 2003b; 2005), by visually counting somata of MOR neurons while classifying them as with or without MOR internalization. This was done using a Zeiss Axio-Imager A1 (Carl Zeiss, Inc., Thornwood, NY) microscope with a fluorescence filter cube for Alexa Fluor 488 (excitation 460–500 nm, beam-splitter 505 nm, emission 510–560 nm; Chroma Technology Corporation, Rockingham, VT) and objectives of 63x (numerical aperture [NA] 1.40) and 100x (NA 1.40). Counting was done blind to the treatment. Neuronal somata with five or more endosomes were considered as having internalization. When necessary, the DAPI staining was used to confirm the presence of a cell soma. All MOR neurons of one dorsal horn were counted for each histological section, and 4 sections per slice (chosen randomly) were used. Typically, this amounted to 70–160 MOR neurons counted per slice. Data from one slice was considered as one replicate measure for statistical purposes.

2.5. Confocal microscopy

Confocal images were acquired at the UCLA's Carol Moss Spivak Cell Imaging Facility using a Leica TCS-SP confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany). Excitation was provided by the 488 nm line of an argon laser, and the emission window was 500–560 nm. For this study we used a pinhole of 1.0 Airy unit and objectives of 20x (NA 0.7)

and 100x oil (NA 1.4), resulting in an estimated optical section thickness (full width at half maximum) of 2.53 μm and 0.62 μm , respectively. Images were acquired as confocal stacks with optical section separations (z-intervals) of 1.18 μm for the 20x objective and 0.203 μm for the 100x objective. Optical sections were averaged 3–4 times to reduce noise. Images were acquired at a digital size of 1024x1024 pixels, using a digital zoom of 2 with the 100x objective. They were later cropped to the relevant part of the field without changing the resolution. Images were processed using Imaris 5.0.2., 64-bit version (Bitplane AG, Saint Paul, MN, www.bitplane.com) and Adobe Photoshop 5.5 (Adobe Systems Inc., Mountain View, CA). Using Imaris, the confocal stacks were examined in 3-dimensions and cropped. For images obtained at 20x, the entire XY field and most of the optical sections were preserved. Images obtained at 100x were cropped in the XY dimension to the cell being examined, and in the Z dimension to optical sections through the middle of the cell. The resulting images were combined into a two-dimensional image and exported to Photoshop, which was used to adjust the contrast using its “adjustment layer/curves” feature, add text, and compose the multi-paneled figures.

2.6. Data analysis

Data were analyzed and graphics composed using Prism 5.1. (GraphPad Software, San Diego, CA). Data are given as the mean $\pm$ standard error (SE) of 3–18 slices. Statistical analyses consisted of one-way ANOVA and Bonferroni's post-test, with significance set at 0.05. The absence of asterisks in the figure indicates that the difference with control is not significant.

Time course data were fitted to a single phase exponential decay function: $Y = (Y_0 - P) \cdot \exp(-X/\tau) + P$. Concentration-response data were fitted by a sigmoidal dose-response function: $Y = \text{bottom} + (\text{top} - \text{bottom}) / (1 + 10^{(\text{Log IC}_{50} - \text{Log X})})$, where “top” and “bottom” are the maximum and minimum values of the response (Y), respectively, and IC_{50} is the concentration (X) that produces half of the maximum inhibition. Parameter constraints were: top < 100%, bottom > 0%. We assumed that concentration-response curves were monotonic and sigmoidal. Baseline measures (zero concentration of drug) were included in the non-linear regression by assigning them a concentration value about three log units lower than the IC_{50} . The statistical error of the IC_{50} was expressed as 95% confidence interval (C.I.). An F-test (Motulsky and Christopoulos, 2003) was used to determine which one of two functions with different number of parameters fitted the data better.

2.7. Chemicals

Actinonin, captopril, clonidine, endomorphin-2, thiorphan and veratridine were purchased from Sigma-RBI (St. Louis, MO). BRL-44408 (2-[2H-(1-methyl-1,3-dihydroisindole)methyl]-4,5-dihydroimidazole maleate), guanfacine, JP-1302 (acridin-9-yl-[4-(4-methylpiperazin-1-yl)-phenyl]amine trihydrochloride hydrate), medetomidine, UK-14304 (brimonidine, 5-bromo-N-(4,5-dihydro-1H-imidazol-2-yl)-6-quinoxalinamine) and rauwolfscine were purchased from Tocris (Ellisville, MO). Isoflurane was from Halocarbon Laboratories, River Edge, NJ.

3. Results

3.1. MOR internalization evoked by veratridine

To evoke the release of opioid peptides, we incubated rat spinal cord slices with 20 μM veratridine for 2 min at 35 $^{\circ}\text{C}$. Veratridine increases neuronal firing by opening voltage-gated Na^{+} channels (Sato and Nakazato, 1991), and has been shown to induce opioid release in the spinal cord (Song and Marvizon, 2003a; 2005; Uzumaki et al., 1984). Opioid release was measured in situ by assessing the internalization of MORs in the somata of dorsal horn neurons, as shown in Fig. 1. Veratridine induced the redistribution of MOR immunoreactivity from the

cell surface to small particles (endosomes) located inside the cell but outside the nucleus (Fig. 1 A). We have shown previously (Song and Marvizon, 2003a) that this veratridine-evoked MOR internalization is due to the release of opioids that bind to the MOR, because it was eliminated by the selective MOR antagonist CTAP and substantially reduced in the presence of low concentrations of Ca^{2+} and in the absence of peptidase inhibitors. To inhibit the enkephalin-cleaving peptidases aminopeptidase N, dipeptidyl carboxypeptidase and neutral endopeptidase, we included in the incubation media their respective inhibitors actinonin, captopril and thiorphan, all at a concentration of 10 μM . Inhibiting these enzymes is required to observe MOR internalization induced by opioid peptides, either released endogenously or added exogenously (Chen et al., 2007b; Song and Marvizon, 2003a). Peptidase inhibitors by themselves do not induce MOR internalization (Chen et al., 2007b; Song and Marvizon, 2003a). In these conditions, veratridine induced MOR internalization in $71 \pm 2\%$ ($n = 39$ slices) of the MOR-immunoreactive neurons in the dorsal horn. The amount of evoked MOR internalization could not be increased by incubating the slices with higher concentrations of veratridine or for longer time periods. Quite the opposite: 100 μM veratridine directly inhibits the MOR internalization mechanism itself (Chen et al., unpublished results).

3.2. Adrenergic α_2 agonists inhibited veratridine-evoked MOR internalization

To determine whether adrenergic α_2 receptors inhibit opioid release in the spinal cord, we investigated the ability of four α_2 agonists to inhibit veratridine-evoked MOR internalization. All agonists inhibited the evoked MOR internalization. Slices were preincubated with the agonists (and peptidase inhibitors) for 5 min before veratridine stimulation. As can be observed in Fig. 2, shorter preincubation times (1 min or 0 min before veratridine) resulted in the loss of the inhibition by the agonist guanfacine (3 μM), whereas a longer preincubation time (10 min) produced the same result as 5 min. This probably reflects the time required for the drugs to penetrate the slices. After veratridine, slices were incubated for 10 min more to allow the completion of the internalization process (Marvizon et al., 1999).

The classic α_2 agonist clonidine (Fig. 3 A) produced an almost complete inhibition of the evoked MOR internalization. Its effect follows a single phase concentration-response curve with an IC_{50} of 1.7 μM ($\log \text{IC}_{50} = -5.76 \pm 0.16$).

Guanfacine, another α_2 adrenergic agonist, also produced a practically complete inhibition of MOR internalization (Fig. 3 B). Its potency was almost one order of magnitude higher than clonidine, with an IC_{50} of 248 nM ($\log \text{IC}_{50} = -6.61 \pm 0.13$). Fig. 1 C shows examples of dorsal horn neurons in a slice stimulated with veratridine in the presence of 3 μM guanfacine: MOR immunoreactivity is present at the cell surface and endosomes are absent from the cytoplasm.

A third α_2 agonist, medetomidine (Fig. 3 C), inhibited the evoked MOR internalization with very high potency ($\text{IC}_{50} = 0.3$ nM, $\log \text{IC}_{50} = -9.50 \pm 0.36$) but only partially: the inhibition reached a plateau at $37 \pm 5\%$ MOR neurons with internalization. These results indicate that medetomidine behaves as a partial agonist of the α_2 receptor that inhibits opioid release. Alternatively, the effect of medetomidine can be interpreted as an initial inhibition phase followed by a reversal of the inhibition at concentrations higher than 3 nM, as indicated by the dotted line in Fig. 3 C (“bell-shaped” curve). However, comparing the fittings to the two functions (i.e., single phase inhibition vs. inhibition followed by reversal) with an F-test revealed that the single phase inhibition provided a better fit of the data ($p=0.279$).

A fourth α_2 agonist, UK-14304, produced an even more complex effect on the evoked MOR internalization (Fig. 2 D): an inhibitory phase up to 30 nM, followed by reversal of the inhibition at 50 nM and 100 nM, and a second inhibition phase at 300 nM and 1 μM . It was not possible to fit all the points to a single function. In view of this, we tentatively fitted the points up to 30

nM to a concentration-response curve, obtaining an approximate IC₅₀ of 22 nM (log IC₅₀ = -7.66 ± 0.46) for the initial inhibitory phase. This complex effect of UK-14304 may be caused by its interaction with receptors other than α_2 adrenergic receptors at its higher concentrations. Fig. 1 B illustrates the inhibition by 30 nM UK-1403 of MOR internalization evoked by veratridine: in five of the six MOR neurons shown MOR immunoreactivity is present at the cell surface and not in endosomes.

3.3. Adrenergic α_2 agonists did not inhibit MOR internalization induced by a MOR agonist

Since MOR internalization evoked by veratridine was due to opioid release, we attributed its inhibition by α_2 agonists to an inhibition of opioid release mediated by α_2 adrenergic receptors. However, the α_2 agonists could have inhibited the MOR internalization process itself. To rule out this possibility, we induced MOR internalization by incubating the slices with the MOR agonist endomorphin-2 in the absence and presence of α_2 agonists. Slices were incubated with the α_2 agonists for 5 min at 35 °C, and then 100 nM endomorphin-2 was added for another 10 min. No peptidase inhibitors were used, because endomorphin-2 is not appreciably degraded by peptidases in these conditions (Song and Marvizon, 2003a). In agreement with our previous results (Song and Marvizon, 2003a), endomorphin-2 induced MOR internalization in most MOR dorsal horn neurons (Fig. 4). The internalization induced by endomorphin-2 was not affected by 3 μ M clonidine, 3 μ M guanfacine, 3 nM medetomidine or 30 nM UK-14304, their most effective concentrations to inhibit of veratridine-evoked MOR internalization (Fig. 3). Therefore, the inhibition of veratridine-evoked MOR internalization by α_2 agonists should be attributed to inhibition of opioid release and not to inhibition of MOR internalization itself.

3.4. Reversal by an α_{2C} antagonist of the inhibition of MOR internalization by α_2 agonists

There are three pharmacologically and genetically distinct subtypes of α_2 adrenergic receptors: α_{2A} , α_{2B} and α_{2C} (Yaksh, 1985). The α_{2C} subtype has been found to colocalize with opioids in presynaptic terminals of intrinsic dorsal horn neurons (Marvizon et al., 2007; Olave and Maxwell, 2002, 2004; Stone et al., 1998) and therefore is likely to mediate the inhibition of opioid release produced by the α_2 agonists. To investigate this possibility, we determined whether the subtype-selective α_{2C} antagonist JP-1203 (Sallinen et al., 2007; Tricklebank, 2007) reversed the inhibition produced by the agonists. There are no subtype-selective α_2 agonists available. Although guanfacine shows some selectivity for α_{2A} receptors (Uhlen et al., 1997), it is entirely possible that at the concentrations we used it, its effect is mediated by α_{2C} receptors. Indeed, as shown in Fig. 5, the inhibition produced by guanfacine (3 μ M) was reversed by the selective α_{2C} antagonist JP-1302 (10 nM), but not by the selective α_{2A} antagonist BRL-44408 (100 nM) (Jurgens et al., 2007). Fig. 1D shows representative dorsal horn neurons in a slice incubated with guanfacine (3 μ M) and JP-1302 (3 nM). Note that MOR internalization is abundant in all neurons, whereas no MOR internalization is observed in a slice incubated with 3 μ M guanfacine alone (Fig. 1 C). JP-1302 (10 nM) also prevented the inhibition produced by 3 μ M clonidine. Similarly, the effect of 30 nM UK-14304 (the concentration that produced the most inhibition of MOR internalization) was prevented by JP-1302 (10 nM) or rauwolscine (30 nM), an α_2 antagonist with little subtype selectivity. These results show that the inhibition of the evoked MOR internalization produced by guanfacine, clonidine and low concentrations of UK-14304 is caused by the activation of α_{2C} receptors.

4. Discussion

Our results demonstrate for the first time the existence of an adrenergic inhibition of opioid release in the spinal dorsal horn, mediated by α_{2C} adrenergic receptors.

4.1. Mechanism

We show that four agonists of α_2 adrenergic receptors - clonidine, guanfacine, medetomidine and UK-14304 - inhibited MOR internalization evoked by veratridine. Since these four agonists did not affect MOR internalization produced by the MOR agonist endomorphin-2, their effect has to be attributed to an inhibition of the opioid release evoked by veratridine. The inhibition produced by the agonists was prevented by the selective α_{2C} antagonist JP-1302, but not by the α_{2A} antagonist BRL-44408. Therefore, the inhibition of opioid release was caused by the activation of α_{2C} receptors.

This conclusion is consistent with previous immunohistochemistry studies demonstrating a substantial colocalization in the dorsal horn of α_{2C} receptors with Met-enkephalin (Olave and Maxwell, 2002, 2004), Leu-enkephalin (Stone et al., 1998) or the pan-opioid antibody 3-E7 (Marvizon et al., 2007). However, α_{2C} receptors did not colocalize with preprodynorphin (Stone et al., 1998), which appears to be expressed in dorsal horn neurons different from the ones expressing preproenkephalin (Cruz and Basbaum, 1985). Therefore, the decrease in evoked MOR internalization produced by α_{2C} agonists was probably caused by an inhibition of enkephalin release, and not of dynorphin release.

The presence of α_{2C} receptors in enkephalin-containing terminals suggests that they inhibit enkephalin release using an intracellular signaling pathway inside the presynaptic terminal. Adrenergic α_2 receptors signal through inhibitory α_i G proteins (Summers and McMartin, 1993). Therefore, the most likely mechanism for the inhibition of release would be the inactivation of voltage-gated Ca^{2+} channels by the $\beta\gamma$ subunits of G proteins (Adamson et al., 1989; Dolphin, 2003; Li and Bayliss, 1998).

4.2. Physiological role

Opioids released in the dorsal horn produces analgesia, mainly by activating MORs (Budai and Fields, 1998; Chen et al., 2007b; Takemori and Portoghese, 1993). Therefore, in situations where endogenous opioids are producing some level of analgesia, the inhibition of opioid release by α_{2C} receptors would be expected to increase pain. This seems to run contrary to the well-established fact that α_2 adrenergic receptors in the spinal cord produce analgesia (Pertovaara, 2006; Yaksh, 1985). This analgesia is largely mediated by the α_{2A} subtype of adrenergic receptors, which inhibit the release of glutamate and substance P from primary afferent terminals (Kamisaki et al., 1993; Kawasaki et al., 2003; Kuraishi et al., 1985; Li and Eisenach, 2001; Pan et al., 2002; Takano et al., 1993). However, there is also evidence that α_{2C} receptors contribute to this analgesic effect (Fairbanks et al., 2002).

Hence, these α_{2C} receptors may play a complex role in modulation of pain in the dorsal horn. As stated above, α_{2C} receptors and enkephalins are present together in the presynaptic terminals of dorsal horn interneurons (Olave and Maxwell, 2002, 2004; Stone et al., 1998). Paradoxically, however, these interneurons are excitatory, and make glutamatergic synapses with nociceptive projection neurons (Olave and Maxwell, 2003a; 2003b). Therefore, despite the fact that they release enkephalins, these interneurons seem to belong to a pro-algesic pathway. It is likely that the α_{2C} receptors, besides inhibiting enkephalin release, also inhibit glutamate release. The resulting decrease in the excitatory input to the projection neurons may be responsible for the analgesic effect of the α_{2C} receptors.

Then, what could be the physiological role of the inhibition of enkephalin release by α_{2C} adrenergic receptors? Noradrenergic terminals in the dorsal horn belong to descending axons that originate in adrenergic nuclei of the brain stem (Pertovaara, 2006). These terminals do not appear to form synapses (Rajaofetra et al., 1992), which implies that norepinephrine acts by volume transmission in the dorsal horn (Zoli and Agnati, 1996). Therefore, whenever

norepinephrine is released in the dorsal horn, it would concurrently produce analgesia by acting on α_{2A} receptors and inhibit enkephalin release by acting on α_{2C} receptors. This could serve to shut down the spinal opioid system whenever the spinal adrenergic system is active. This may be a way to avoid the excessive analgesia that could ensue if both the adrenergic and the opioid systems in the spinal cord are active at the same time.

4.3. Relationship with other neurotransmitter receptors that modulate spinal opioid release

Adrenergic α_{2C} receptors are in no way unique in their ability to inhibit opioid release. In a previous study (Song and Marvizon, 2005), we showed that spinal opioid release is inhibited by NMDA receptors acting in conjunction with large conductance Ca^{2+} -sensitive K^+ (BK) channels. It is likely that these NMDA receptors and BK channels are extrasynaptic (Isaacson and Murphy, 2001), and not presynaptic like the α_{2C} receptors. Therefore, these two modulatory mechanisms probably act independently.

More recently, we showed that spinal opioid release is inhibited by serotonin 5-HT_{1A} receptors (Song et al., 2007). However, the 5-HT_{1A} agonist 8-hydroxy-DPAT inhibited only half of the evoked MOR internalization, suggesting that these receptors inhibit release from only some of the opioid terminals. Alternatively, inhibition by 5-HT_{1A} receptors may require an extracellular signal. In contrast, we found that opioid release is not affected by GABA_A, GABA_B, cholecystokinin or δ -opioid receptors (Song and Marvizon, 2005). A substantial challenge for future studies will be to explain how these different neurotransmitter systems interact with each other to modulate spinal opioid release.

4.4. Therapeutic implications

It may be possible to produce analgesia by increasing the availability of opioid peptides in the spinal cord. In fact, blocking peptidase degradation of opioids produced analgesia (Chou et al., 1984; Fournie-Zaluski et al., 1992; Noble et al., 1992b), with the added benefit of producing little tolerance and dependence (Noble et al., 1992a; Noble et al., 1992c). Another way to increase the availability of opioids in the spinal cord would be by blocking neurotransmitter receptors that inhibit their release. However, as explained above, blocking α_{2C} receptors is unlikely to produce analgesia because the disinhibition of glutamate release onto nociceptive neurons would probably cancel the analgesic effect produced by the increase in opioid release. Something different may happen in situations like inflammation, where there is an increased activity of descending noradrenergic pathways (Pertovaara, 2006). In these cases inhibiting α_{2C} receptors would increase the release of opioids, which then would act synergistically with α_{2A} receptors (Monasky et al., 1990; Ossipov et al., 1990; Stone et al., 1997b) to increase the analgesia. Further studies using in vivo pain models are needed to address this issue.

Acknowledgements

Supported by NIDA grant 2 R01 DA012609 to J.C.M. Confocal images were acquired at Carol Moss Spivak Cell Imaging Facility of the Brain Research Institute at UCLA, with the assistance of Dr. Matthew J. Schibler. We thank Orlando Perez for his technical help.

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Figure 1. Images of MOR neurons in the dorsal horn after evoking opioid release with veratridine Spinal cord slices were incubated at 35 °C with 20 μ M veratridine, peptidase inhibitors (actinonin, captopril and thiorphan, 10 μ M) and the following α 2 adrenergic compounds: **A**, none; **B**, the agonist UK-14,304 (30 nM); **C**, the agonist guanfacine (3 μ M); **D**, guanfacine (3 μ M) and the antagonist JP-1203 (3 nM). Opioid release resulted in the internalization of MORs in dorsal horn neurons, visualized with immunofluorescence. Abundant MOR internalization is observed in the control (A) and after guanfacine plus the antagonist JP-1203 (D), but little internalization was evoked in the presence of the agonists UK-14,304 (B) or guanfacine (C). Main panels show most of the dorsal horn: images were taken with a 20x objective (scale bars are 50 μ m) and consist of 4 confocal sections separated 1.18 μ m taken through the middle of the histological section. Insets show examples of MOR neurons: images were taken with a 100x objective and a digital zoom of 2 (scale bars are 5 μ m) and consist of 4–12 optical sections spaced 0.20 μ m taken through the middle of the cells.

Figure 2. Effect of preincubation time with guanfacine

Spinal cord slices were pre-incubated for 10 min with peptidase inhibitors (actinonin, captopril and thiorphan, 10 μ M), and with 3 μ M guanfacine for the pre-incubation times indicated in the X-axis. Then, opioid release was evoked with 20 μ M veratridine for 2 min. Slices were kept in aCSF containing peptidase inhibitors for 10 more minutes before fixing. The dotted line indicates the amount of MOR internalization obtained in the absence of guanfacine. The curve represents fitting the points to an exponential decay function (half-life = 2 min).

Figure 3. Inhibition of opioid release by adrenergic α_2 agonists

Opioid release and subsequent MOR internalization was evoked by stimulating spinal cord slices with 20 μM veratridine in the presence of peptidase inhibitors (actinonin, captopril and thiorphan, 10 μM). Concentration-response curves were obtained for the α_2 adrenergic agonists clonidine (A), guanfacine (B), medetomidine (C), and UK-14304 (D). Data points give the mean $\pm$ SEM of 3–18 slices. ANOVA and Bonferroni's post-test were used to determine whether there were significant differences with the controls (zero concentration of agonist): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Curves represent fitting of the data to a sigmoidal dose-response function. (A) Clonidine produced a total inhibition with an IC_{50} of 1.7 μM (95% CI 0.8 – 3.7 μM). (B) Guanfacine produced a total inhibition with an IC_{50} of 248 nM (95% CI 131 – 67 nM). (C) Medetomidine produced a partial inhibition that leveled off at $37 \pm 5\%$, with an IC_{50} of 0.3 nM (95% CI 0.06 – 1.7 μM). The dotted line represents alternative fitting of the data to a bell-shaped curve: inhibition ($\log IC_{50} = -8.96 \pm 0.84$) followed by reversal of the inhibition ($\log EC_{50} = -8.02 \pm 1.53$). However, an F-test revealed that the bell-shaped curve did not provide a better fit of the data ($p = 0.279$). (D) UK-14304 produced a complex dose-response consisting of an inhibition phase up to 30 nM followed by reversal of the inhibition at 100 nM and a second inhibition phase. Fitting the points up to 30 nM UK-14304

to a dose-response function yielded an IC₅₀ of 22 nM (95% CI 2.5 – 189 nM). Points at higher concentrations are linked by a dotted line.

Figure 4. Adrenergic α_2 agonists did not inhibit MOR internalization induced by endomorphin-2 MOR internalization was evoked by stimulating spinal cord slices with the MOR agonist endomorphin-2 (100 nM) for 10 min. The α_2 agonists were added 5 min prior to endomorphin-2 at concentrations that produced maximum inhibition of veratridine-evoked MOR internalization: clonidine 3 μ M, guanfacine 3 μ M, medetomidine 3 nM, UK-14304 30 nM. ANOVA revealed no significant differences with endomorphin-2 alone ($p=0.79$).

Figure 5. Inhibitions of evoked MOR internalization by clonidine, guanfacine or UK-14304 were prevented by the α_{2C} antagonist JP-1302

Opioid release and subsequent MOR internalization was evoked by stimulating spinal cord slices with 20 μ M veratridine in the presence of peptidase inhibitors (actinonin, captopril and thiorphan, 10 μ M). Clonidine (3 μ M), guanfacine (3 μ M) or UK-14304 (30 nM) inhibited the evoked MOR internalization. The selective α_{2C} antagonist JP-1302 (10 nM), applied together with clonidine, guanfacine or UK-14304, prevented the inhibition of MOR internalization. JP-1302 did not have any effect by itself. The selective α_{2A} antagonist BRL-44408 (100 nM) did not reverse the inhibition produced by guanfacine. Inhibition by UK-14304 was also prevented by the α_2 antagonist rauwolscine (30 nM). Numbers inside the bars indicated the number of slices (n) in each group. Statistical significance was determined with one-way ANOVA (overall $p < 0.0001$) and Bonferroni's post-test: *** $p < 0.001$ compared to control; ††† $p < 0.001$, † $p < 0.05$, for the comparisons indicated.

Figure 6.