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Ability of veratridine and high potassium to produce μ -opioid receptor internalization by evoking the release of opioid peptides in the rat spinal cord

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Abstract

Veratridine and high K^+ evoked opioid release in rat spinal cord slices, measured by μ -opioid receptor (MOR) internalization. Veratridine elicited up to 75% MOR internalization with an atypical concentration-response: its effect appeared abruptly from 5 μ M to 10 μ M, and declined thereafter to disappear at 100 μ M. Veratridine at 100 μ M also abolished MOR internalization induced by an exogenous MOR agonist, endomorphin-2, showing that its inhibitory phase was caused by direct inhibition of MOR internalization. In contrast, veratridine elicited neurokinin 1 receptor internalization (used to measure substance P release) following a monophasic dose-response curve. KCl evoked up to 80% MOR internalization following a monophasic, steep dose-response curve with an EC_{50} of 19 mM. K^+ -evoked MOR internalization disappeared in the presence of lidocaine or in the absence of peptidase inhibitors, indicating that it involved neuronal firing and peptide release. Veratridine and high K^+ produced substantially more MOR internalization when applied for 2 min than for 20 min, because of a direct inhibition of MOR internalization with the longer incubation times. These results show that short incubations with 20 μ M veratridine or 30 mM KCl are optimal stimuli to evoke MOR internalization in the spinal cord.

Keywords

Depolarization; dynorphin; enkephalin; internalization; Na^+ channels; potassium; substance P; veratridine

Introduction

Endogenous opioid peptides (henceforth, “opioids”) released in the spinal cord induce analgesia by activating μ -opioid receptors (MORs) and δ -opioid receptors (Budai and Fields, 1998; Chen et al., 2007; Takemori and Portoghese, 1993). Understanding the neuronal circuitry and pharmacological mechanisms that control opioid release is important to clarify the role of opioids in conditions that produce analgesia, such as stress (Yamada and Nabeshima, 1995), acupuncture (Han, 2003) or pain (Gear et al., 1999).

Most studies on opioid release from the spinal cord have used immunoassays detecting just one opioid, typically Met-enkephalin (Bourgoin et al., 1990; Cesselin et al., 1989; Le Bars et al., 1987; Tseng et al., 1985) or dynorphin A (Leitermann et al., 2004). However, the spinal cord contains and releases many other opioids (Yaksh et al., 1983). Dorsal horn neurons express the pre-proenkephalin and pre-prodynorphin genes (Todd and Spike, 1993) - but not the pro-opiomelanocortin gene (Tsou et al., 1986). Sequential cleavage of the opioid precursor proteins produces many different peptides of different lengths, all of which are able to activate opioid receptors. Therefore, measuring the release of just one peptide is a poor predictor of opioid receptor activation. An approach that solves this problem is the use of MOR internalization as an in situ measure of opioid release, since it detects all opioid peptides able to activate this receptor (Eckersell et al., 1998; Mills et al., 2004; Song and Marvizon, 2003a; 2003b; Trafton et al., 2000). Although alkaloid opiates like morphine can activate the MOR without inducing its internalization (Keith et al., 1998; Keith et al., 1996), all naturally occurring opioids produce MOR internalization (Song and Marvizon, 2003a). MOR internalization correlates well with opioid-induced analgesia and other measures of MOR activation (Chen et al., 2007; Marvizon et al., 1999b; Trafton et al., 2000). However, naturally occurring opioids need to be protected from peptidase degradation in order to induce MOR internalization (Chen et al., 2007; Song and Marvizon, 2003a).

Many previous studies were conducted in vivo, measuring opioid release in response to noxious stimuli (Bourgoin et al., 1990; Cesselin et al., 1989; Le Bars et al., 1987) or sciatic nerve stimulation (Yaksh et al., 1983). However, in vitro measures of opioid release or MOR internalization offer distinctive advantages when the goal is to investigate the molecular mechanisms that regulate release. Thus, spinal cord slices can be used to measure opioid release under well-defined conditions, including drug concentration, incubation time, and chemical or electrical stimulation. Using this approach we discovered that spinal opioid release is inhibited by 5-HT_{1A} serotonin receptors (Song et al., 2007), and by NMDA receptors acting in conjunction with large conductance K⁺ channels (Song and Marvizon, 2005).

One important issue in these experiments is the type of stimulus used to evoke opioid release from the slices. Electrical stimulation the dorsal root is very effective to induce substance P release, but it does not induce opioid release (Song and Marvizon, 2003b) because primary afferents do not contain opioids, at least in their central terminals (Bras et al., 2001; Carlton and Coggeshall, 1997; Pohl et al., 1994). Likewise, incubating slices with capsaicin evoked abundant substance P release (Lao et al., 2003), but not opioid release (Song and Marvizon, 2003b).

So far, we have found three stimuli that are very effective in evoking opioid release and MOR internalization in spinal cord slices: electrical stimulation of the dorsal horn, high K⁺ and veratridine (Song and Marvizon, 2003a; Song and Marvizon, 2003b, 2005). Using high K⁺ or veratridine appear to have some advantages over electrical stimulation to evoke opioid release, but in past studies we did not identify the optimal conditions for the use of these stimuli. This was the goal of the present study.

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.

Spinal cord slices

Coronal spinal cord slices were prepared as previously described (Marvizon et al., 1999b; Song and Marvizon, 2003a; 2003b; 2005). Media used to prepare the slices were: aCSF, containing

(in mM) 124 NaCl, 1.9 KCl, 26 NaHCO₃, 1.2 KH₂PO₄, 1.3 MgSO₄, 2.4 CaCl₂ 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, using maximum vibration and minimum advance speed. Up to six slices from each animal were cut sequentially in the L1-L4 region.

Slice treatment

To evoke opioid release and subsequent MOR internalization, slices were incubated in aCSF containing peptidase inhibitors and different concentrations of veratridine or KCl. In previous studies (Chen et al., 2007; Song and Marvizon, 2003a; 2005), we found that peptidase inhibitors were required for the induction of MOR internalization by naturally-occurring opioids. Peptidase inhibitors were 10 µM actinonin or amastatin (to inhibit aminopeptidases), 10 µM captopril (to inhibit dipeptidyl carboxypeptidase) and 10 µM thiorphan or phosphoramidon (to inhibit neutral endopeptidase). Phosphoramidon was supplemented with 6 µM dithiothreitol as a preservative. Slices were placed on a nylon net glued to a plastic ring suspended halfway inside a tube or small beaker containing aCSF at 35 °C and superficially gassed with a flow of 95% O₂ / 5% CO₂. With short (2 min) exposures to veratridine or KCl, slices were incubated with peptidase inhibitors starting 5 min before and ending 10 min after the stimuli. This allowed enough time for the peptidase inhibitors to enter the slice and for MOR internalization to be completed (Trafton et al., 2000). With long (20 min) exposures to veratridine or KCl, peptidase inhibitors were applied together with them. At the end of the incubations, the slices were fixed by immersion in ice-cold fixative (4 % paraformaldehyde, 0.18 % picric acid in 0.1 M sodium phosphate buffer).

Immunohistochemistry

Histological sections of 25 µm were cut from the slices and labeled as previously described (Marvizon et al., 1999b; Song and Marvizon, 2003a; 2003b; 2005). The sections were either single-labeled with a rabbit antibody against MOR or double-labeled using this antibody and a guinea pig antibody against the NK1R. The rabbit MOR antiserum (1:7000 dilution) was raised against amino acids 384–398 of the cloned rat MOR-1 (DiaSorin, Stillwater, MN, catalog no. 24216). It was 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 guinea pig NK1R antiserum (1:1000 dilution) was raised against amino acids 393–407 of the rat NK1R (Chemicon, Temecula, CA, catalog # AB5800). Double labeling with the guinea pig NK1R antiserum and a rabbit NK1R antiserum (# 94168, a gift from Dr. Nigel Bunnett, UCSF), raised against the same epitope and previously characterized (Grady et al., 1996), resulted in complete co-localization of the staining at the cellular and sub-cellular levels.

The slices were fixed, cryoprotected, frozen on dry ice, and sectioned with a cryostat at 25 µm in the coronal plane. Sections were washed twice with PBS, and twice with PBS, 0.3% Triton X-100, 0.001% thimerosal (PBS/Triton) containing 5% normal goat serum, and then incubated at room temperature overnight with the MOR and NK1R primary antibodies in PBS/Triton. After three washes with PBS, sections were incubated for two hours at room temperature with the secondary antibodies (combined for double labeling) in PBS/Triton. Secondary antibodies were Alexa-488 goat anti-rabbit IgG and Alexa-568 goat anti-guinea pig IgG, both from Molecular Probes, used at 1:2000 and 1:1000 dilutions, respectively. Sections were washed four more times with PBS and mounted in Prolong or Prolong Gold (Molecular Probes).

Quantification of MOR and NK1R internalization

Previously described procedures were used to quantify MOR internalization (Marvizon et al., 1999b; Song and Marvizon, 2003a; 2003b; 2005) and NK1R internalization (1999a; Marvizon et al., 1997; 2003). Briefly, MOR or NK1R neurons were visually counted while classifying them as with or without internalization. This was done using a Zeiss Axiovert 135 (Carl Zeiss, Inc., Thornwood, NY) microscope with an objective of 100× (numerical aperture 1.40). Counting was done blind to the treatment. Neuronal somata with >5 endosomes for MORs and >10 endosomes for NK1Rs were considered as having internalization. All MOR or NK1R neurons of one dorsal horn were counted for each histological section, and 4 sections per slice (chosen randomly) were used. Data from one slice was considered as one replicate measure for statistical purposes.

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 for the green signal and the 561 nm line of a diode laser for the red signal. Corresponding emission windows were 500–560 nm and 600–670 nm. We used a pinhole of 1.0 Airy unit and objectives of 63× oil (NA 1.32) and 100× oil (NA 1.4), resulting in an estimated optical section thickness (full width at half maximum) of 0.69 μm and 0.62 μm, respectively. Images were acquired as confocal stacks with optical section separation (z-interval) of 0.57 μm. Optical sections were averaged 3–4 times to reduce noise. Images were acquired at a digital size of 1024×1024 pixels, and were later cropped to the relevant part of the field without changing the resolution. Images were processed using Imaris 5.7.1., 64-bit version (Bitplane AG, Saint Paul, MN) and Adobe Photoshop 5.5 (Adobe Systems Inc., Mountain View, CA). With Imaris the confocal stacks were examined, cropped in 3-dimensions and combined into a two-dimensional image. This image was exported to Photoshop, which was used to adjust the gamma, add text, and compose the multi-paneled figure.

Data analysis

Data were analyzed and graphics composed using Prism 5.1. (GraphPad Software, San Diego, CA). Data are the mean ± standard error (SE) of 3–8 slices. Statistical analyses consisted of a t-test or one-way ANOVA followed by 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.

Veratridine concentration-response data were fitted by a dose-response function with variable Hill slope (n_H): $Y = \text{bottom} + (\text{top} - \text{bottom}) / (1 + 10^{-(\text{Log EC}_{50} - X) * n_H})$; where “top” and “bottom” are the maximum and minimum values of the response, respectively, and EC_{50} is the concentration (X) that produces half of the effect. Parameter constraints were: top < 100%, bottom > 0%. KCl concentration-response data were fitted by a binding saturation function with variable n_H : $Y = B_{\text{max}} * X^{n_H} / (K_D^{n_H} + X^{n_H})$. “Global fitting” of the data to two functions, one with n_H constrained to 1 and another with variable n_H , was used to determine which of these two models provided the best fitting to the data according to Akaike's Information Criterion (Motulsky and Christopoulos, 2003).

Results

We studied the ability of two chemical stimuli, veratridine and high K^+ (KCl), to evoke the release of opioids peptides in spinal cord slices. MOR internalization was used as an in situ measure of opioid release. In previous studies (Chen et al., 2007; Song and Marvizon, 2003a; 2003b; 2005) we found that naturally occurring opioids can only induce MOR internalization if they are protected from peptidase degradation. Accordingly, all the experiments described

here were done in the presence of peptidase inhibitors (see “Slice treatment” in the Methods section).

Concentration-response of veratridine to produce MOR internalization

Veratridine increases Na^+ fluxes through voltage-dependent Na^+ channels (Sato and Nakazato, 1991). We have previously shown (Song and Marvizon, 2003a; Song and Marvizon, 2005) that incubating spinal cord slices with veratridine induces MOR internalization. To determine the optimal concentration of veratridine to induce MOR internalization, we incubated spinal cord slices with different concentrations of veratridine up to 100 μM . Since veratridine is also likely to induce the release of other neuropeptides, we measured the release of substance P in the same slices through the internalization of neurokinin 1 receptors (NK1Rs). Sections from the slices were double-labeled with MOR and NK1R antibodies as shown in Fig. 1. MORs and NK1Rs were always present in different neurons, as previously reported (Song and Marvizon, 2003b; Spike et al., 2002). Interestingly, some NK1R neurons had clusters of MOR immunoreactive puncta in close apposition to their soma (Fig. 1 A, upper middle), which are likely MOR-containing primary afferent terminals (Abbadie et al., 2001; Besse et al., 1990; Fields et al., 1980).

The concentration-response of veratridine to produce MOR internalization was quite atypical (Fig. 2 A). Veratridine did not evoke any MOR internalization at concentrations of 1 μM , 3 μM or 5 μM , but at 10 μM it induced internalization in 75 % of the MOR neurons. MOR internalization then decreased with the concentration of veratridine, practically disappearing with 100 μM veratridine. In contrast, veratridine stimulated NK1R internalization following a monophasic dose-response curve (Fig. 2 B), with no inhibitory phase found up to 100 μM . The potency of veratridine to evoke NK1R internalization ($\text{EC}_{50} = 12 \mu\text{M}$) was somewhat lower than its potency to induce MOR internalization ($\text{EC}_{50} = 6.7 \mu\text{M}$). The dose-response curve for veratridine to evoke NK1R internalization was steep ($n_H = 3.8 \pm 2.9$) although less so than the facilitatory phase of the effect of veratridine.

Fig. 1 shows images of MOR and NK1R neurons in the superficial dorsal horn (laminae I-II) representative of these findings. Fig. 1 A shows three MOR neurons in a slice incubated with 3 μM veratridine, none of them with receptor internalization; the one NK1R neuron in this image does show some internalization. Fig. 1 B corresponds to a slice incubated with 10 μM veratridine: all three MOR neurons and two out of three NK1R neurons have internalization. In Fig. 2 C the concentration of veratridine was 100 μM : all three MOR neurons lack internalization, but three out of four NK1R neurons have it.

Veratridine at 100 μM directly inhibits MOR internalization

Veratridine induces MOR internalization by evoking the release of endogenous opioids, that then bind to the receptor and cause its internalization (Song and Marvizon, 2003a). However, it is possible that the inhibitory phase observed with high concentrations of veratridine was caused by its direct inhibition of the receptor internalization mechanism. To investigate this possibility, we induced MOR internalization by incubating the slices with an exogenous agonist, endomorphin-2, and determined whether 100 μM veratridine was able to inhibit it. Fig. 3 shows that this is indeed the case: whereas 1 μM endomorphin-2 induced internalization in 100% of the MOR neurons, in the presence of 100 μM veratridine MOR internalization was reduced to control levels.

Concentration-response of KCl to produce MOR internalization

We also measured the amount of MOR internalization produced by different concentrations of KCl. The concentration of KCl in aCSF was increased from its value of 1.9 mM in normal aCSF up to 70 mM, decreasing the concentration of NaCl by the same amount in order to

maintain the osmolarity. As can be seen in Fig. 4, the evoked MOR internalization increased steeply from practically none at 10 mM KCl to about 80 % at 30 mM KCl. At 50 mM and 70 mM, KCl did not produce any further increases in MOR internalization. Fitting of a binding saturation function with variable Hill slope (n_H) to the data yielded a B_{max} of 81 ± 7 %, and a K_D of 19 ± 1 mM. The value of n_H was high but indeterminate because it reached its constrain value, set at 5.

To confirm that MOR internalization evoked by 30 mM KCl was caused by opioid release, we omitted peptidase inhibitors from the medium. As shown in Fig. 5, this resulted in a marked and significant reduction in MOR internalization. The Na^+ channel blocker lidocaine (1 mM) reduced MOR internalization evoked by 30 mM KCl (in the presence of peptidase inhibitors, Fig. 5) to near control levels. This is consistent with the idea that 30 mM KCl induces opioids release by increasing the firing of action potentials in the opioid-expressing neurons.

Time of incubation with veratridine or high K^+

To determine the optimal time for veratridine to induce MOR internalization, we incubated the slices with 30 μ M veratridine for 2 min or 20 min. As shown in Fig. 6 A, the shorter incubation time produced significantly more MOR internalization. In two additional slices incubated with 30 μ M veratridine for 10 min, the amount of MOR internalization was the same as with 20 min (36 % and 33 %). Similarly, a 2 min incubation with 50 mM KCl produced more MOR internalization than a 20 min incubation (Fig. 6 B).

A possible explanation of the reduced MOR internalization produced by KCl at 20 min is that long depolarizations inhibit the MOR internalization process. To investigate this possibility, we induced MOR internalization directly by incubating the slices for 20 min with exogenous endomorphin-2 (1 μ M), with or without 50 mM KCl. Endomorphin-2 induced MOR internalization in 100 % of the neurons, but the presence of 50 mM KCl dramatically reduced the amount of MOR internalization. Therefore, long incubation with high K^+ do inhibit the MOR internalization mechanism.

Discussion

The main findings of this study are: 1) veratridine and KCl are effective stimuli to evoke opioid release and subsequent MOR internalization in the spinal cord; 2) they show steep concentration-responses to induce MOR internalization; 3) high concentrations of veratridine inhibited MOR internalization directly; 4) prolonged incubations with KCl also inhibited MOR internalization.

Opioid release evoked by veratridine

In a previous report (Song and Marvizon, 2003a) we showed that MOR internalization evoked by veratridine is abolished by a MOR antagonist, by low Ca^{2+} , and in the absence of peptidase inhibitors. This indicates that veratridine elicits the release of opioids, which then bind to MORs to induce their internalization. Opioid release is likely driven by the rapid firing of action potentials when veratridine causes the opening of voltage-dependent Na^+ channels (Dargent et al., 1996; Pauwels et al., 1990; Satoh and Nakazato, 1991). In other studies, veratridine was found to evoke the release of enkephalin (Uzumaki et al., 1984) and dynorphin (Przewlocka et al., 1990) from the spinal cord, measured with immunoassay.

The concentration-response of veratridine to evoke MOR internalization was remarkably steep, going from almost no internalization with 5 μ M veratridine to peak internalization with 10 μ M veratridine. Elsewhere (Song and Marvizon, 2003b) we have shown that MOR internalization can also be evoked in spinal cord slices by electrical stimulation of the dorsal

horn. This effect was markedly frequency-dependent, with MOR internalization first appearing at 30 Hz and reaching its maximum at 100–500 Hz. This indicates that opioid release occurs when the neurons that express them fire action potentials at high rates, a property common to the release of many neuropeptides (Bartfai et al., 1986; Brezina et al., 2000; Muschol and Salzberg, 2000; Verhage et al., 1991). This frequency-dependence of opioid release could explain the steepness of the concentration-response of veratridine: it is likely that the opioid-releasing neurons respond to rising doses of veratridine by gradually increasing their firing rate, and that only when it crosses a certain threshold does opioid release occur. This would lead to an abrupt transition to opioid release over a narrow concentration range of veratridine. The effect of veratridine on NK1R internalization was also steep (although somewhat less so), probably because substance P release is also frequency-dependent (Go and Yaksh, 1987; Marvizon et al., 1999a; Marvizon et al., 1997).

High concentrations of veratridine inhibit MOR internalization

The lack of MOR internalization observed after incubating the slices with a high concentration of veratridine (100 μ M) was caused by a direct inhibition of MOR internalization. This was shown by the fact that 100 μ M veratridine abolished MOR internalization induced by an exogenous agonist, endomorphin-2. The mechanism of this inhibition probably involves blockade of MOR internalization by the high intracellular levels of Na^+ resulting from the opening of Na^+ channels by veratridine. The Na^+ ionophore monensin, which also promotes Na^+ entry into the cell, inhibits the recycling of internalized receptors (Martin-Negrier et al., 2006), but inhibition of receptor internalization by high intracellular Na^+ has not been reported. An alternative explanation is that 100 μ M veratridine produced neurotoxic effects (Dargent et al., 1996; Pauwels et al., 1990). However, neurotoxic effects usually require hours of exposure to these concentration of veratridine, whereas we incubated the slices with veratridine for just 2 min. Furthermore, we have observed that neuronal death in the slices leads to poor staining with antibodies against NK1Rs or MORs: the immunoreactivity appears as a smear over the whole cell instead of localizing crisply at the surface or in endosomes. In contrast, after exposure to 100 μ M veratridine both the MOR and the NK1R neurons appeared crisply labeled (Fig. 2 C), indicating that the neurons survived the treatment with veratridine. Yet another explanation is that high concentrations of veratridine released substances that inhibit MOR internalization. Interestingly, NK1R internalization was not inhibited by high concentrations of veratridine, probably reflecting differences between the MOR and NK1R neurons, or in the internalization mechanisms of these two receptors.

Opioid release evoked by KCl

We previously found (Song and Marvizon, 2003a) that 50 mM KCl induced MOR internalization in dorsal horn neurons. As in the case of veratridine, MOR internalization induced by KCl was abolished by a MOR antagonist and by low Ca^{2+} . Here we show that it is also substantially reduced in the absence of peptidase inhibitors and in the presence of the Na^+ channel blocker lidocaine. All this is consistent with a mechanism in which KCl depolarizes the opioid-containing neurons, causing them to fire action potentials. This evokes opioid release from their terminals, which in turn activates MORs and causes their internalization. It is a well-known fact that high K^+ concentrations depolarize neurons and makes them discharge action potentials, driving neurotransmitter release (Matthews, 1996). In this case, MOR internalization occurred at moderate concentrations of KCl: it was significant with 20 mM KCl and maximal with 30 mM KCl. Some studies have used KCl concentrations as high as 80 mM to induce neuropeptide release (Del Bianco et al., 1991). This suggests that the opioid-releasing neurons have relatively high K^+ permeability and/or a low resting membrane potential, so that relatively small changes in extracellular K^+ levels make them fire at high rates. MOR internalization increased steeply with the concentration of KCl, but not as

steeply as with veratridine. Again, this could be explained by the fact that opioid-containing neurons need to achieve a high firing rate before they release opioids.

Veratridine and KCl were more effective with short incubation times

Paradoxically, both veratridine and KCl produced more MOR internalization with short than with long incubation times. It is possible that the discharge of action potentials evoked by veratridine and KCl does not last much longer than one or two minutes, so long incubation times do not lead to more opioid release. But then the amount of MOR internalization after 20 min should have been the same as after 2 min, not smaller. The most likely explanation for this paradox would be a direct inhibition of MOR internalization with prolonged incubation times. Indeed, we show that the presence of 50 mM KCl for 20 min inhibited MOR internalization evoked by an exogenous agonist. This finding and the inhibition produced by high concentrations of veratridine indicate that MOR internalization is inhibited by the massive entry of Na⁺ into the MOR neurons resulting from the excessive opening of the Na⁺ channels. Interestingly, high concentrations of veratridine did not inhibit NK1R internalization, suggesting that the NK1R neurons have less Na⁺ channels or more efficient Na⁺ pumps than the MOR neurons. Alternatively, the NK1R internalization mechanism could be less sensitive to Na⁺ than the MOR internalization mechanism.

Which is the best stimulus to evoke opioid release?

In practical terms, KCl has several advantages over veratridine as a stimulus to induce opioid release and MOR internalization. First, only moderate concentrations of KCl (20–30 mM) are required, reducing the possibility of artifacts. Second, unlike veratridine, KCl did not inhibit MOR internalization at high concentrations. Third, the concentration-response of KCl was not as steep as that of veratridine. This allows working with a KCl concentration (20 mM) at which opioid release is sub-maximal in order to study facilitation of opioid release by neurotransmitter receptors. Fourth, KCl is less likely than veratridine to produce neurotoxic effects (Dargent et al., 1996; Pauwels et al., 1990). Fifth, KCl is cheap, stable and easy to handle, whereas veratridine is not readily soluble in water and has complex chemical properties. Still, veratridine has the advantage of evoking little release of substance P (and possibly other neuropeptides) from primary afferents at the concentrations at which it releases opioids. However, this can also be achieved by using KCl in the presence of an inhibitor of substance P release like baclofen (Malcangio and Bowery, 1993; Marvizon et al., 1999a), which does not inhibit opioid release (Song and Marvizon, 2005),

Another stimulus that we have used to evoke opioid release is electrical stimulation of the dorsal horn (Song and Marvizon, 2003b, 2005). It has the advantages over chemical stimuli like veratridine and KCl that it allows to control more factors that influence release. Thus, adjusting the intensity and duration of electrical pulses may serve to recruit different populations of neurons, and using different stimulation frequencies provides information on the firing patterns that are more efficacious to evoke opioid release. Disadvantages of using electrical stimulation include the complexity of the positioning of the electrode relative to the slice, which may lead to different amounts of current crossing the dorsal horn and thus to inconsistent results. In addition, high frequencies of stimulation are required to evoke opioid release (Song and Marvizon, 2003b), which may raise doubts about its physiological relevance.

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Figure 1. Images of MOR and NK1R neurons in the dorsal horn after incubating slices with veratridine

Spinal cord slices were incubated at 35 °C for 2 min with peptidase inhibitors and different concentrations of veratridine. Sections from the slices were double-labeled for MORs (green) and NK1Rs (red). All images consist of two confocal sections separated 0.57 μm . Arrows indicate cells with internalization and arrowheads cells without internalization. A, veratridine 3 μM : image taken with a 100 $\times$ objective showing three MOR neurons without internalization and one NK1R neuron with some internalization. Green dots next to the NK1R neuron are probably presynaptic terminals with MORs. B, veratridine 10 μM : image taken with a 63 $\times$ objective showing three MOR neurons with internalization and two NK1R neurons, one with

internalization and another without. C, veratridine 100 μ M: image taken with a 63 $\times$ objective showing three MOR neurons without internalization and four NK1R neurons, three with internalization and one without. Scale bars are 10 μ m.

Figure 2. Concentration-responses of veratridine to evoke MOR and NK1R internalization

Spinal cord slices were incubated at 35 °C for 2 min with peptidase inhibitors and different concentrations of veratridine. Sections from the slices were double-labeled for MORs and NK1Rs. Data points are the mean $\pm$ SEM of 3–8 slices. **A**, MOR internalization evoked by veratridine. The dotted line curve represents fitting of a dose-response function with variable Hill slope (n_H , constrained to <5) to the MOR internalization data obtained with concentrations of veratridine less than 30 μ M. The fitting yielded the following parameter values: bottom = 5 ± 3 %, top = 82 ± 6 %, Log $EC_{50} = -5.17 \pm 0.04$ ($EC_{50} = 6.7$ μ M), $n_H = 5$ (constrain value). **B**, NK1R internalization evoked by veratridine. The curve represents the fitting of a dose-response function to the NK1R internalization data, which yielded the following parameter values: bottom = 17 ± 5 %, top = 73 ± 10 %, Log $EC_{50} = -4.93 \pm 0.10$ ($EC_{50} = 12$ μ M), $n_H = 3.8 \pm 2.9$. Akaike's Information Criterion revealed that a function with variable n_H fitted the data significantly better (70 % probability) than a function with $n_H = 1$. ANOVA revealed a significant effect of veratridine ($p < 0.0001$ for MOR internalization, $p = 0.0005$ for NK1R internalization). Bonferroni's post test: ** $p < 0.01$, *** $p < 0.001$ compared with 0 M veratridine.

Figure 3. Inhibition by 100 μ M veratridine of MOR internalization induced by endomorphin-2 Spinal cord slices were incubated at 35 °C for 12 min with peptidase inhibitors alone (control) or with 1 μ M endomorphin-2. Veratridine (100 μ M) was added for the first 2 min of the incubation when indicated. Data points are the mean $\pm$ SEM of 3–4 slices. ANOVA revealed significant differences ($p < 0.0001$); Bonferroni's post test: *** $p < 0.001$ compared with control.

Figure 4. Concentration-response of KCl to evoke MOR internalization

Spinal cord slices were incubated at 35 °C for 2 min with peptidase inhibitors and different concentrations of KCl, prepared by iso-osmotic replacement of NaCl in aCSF. Data points are the mean $\pm$ SEM of 3–4 slices. The curve represents the fitting of a binding saturation function with n_H (constrained to <5) to the data, which yielded the following parameter values: $B_{max} = 81 \pm 7\%$, $K_D = 19 \pm 1$ mM, $n_H = 5$ (constrain value). Akaike's Information Criterion revealed that a function with variable n_H fitted the data significantly better (99.9 % probability) than a function with $n_H = 1$. ANOVA revealed a significant effect of KCl ($p < 0.0001$). Bonferroni's post test: ** $p < 0.01$, *** $p < 0.001$ compared with 0 mM KCl.

Figure 5. MOR internalization evoked by KCl was inhibited by lidocaine and in the absence of peptidase inhibitors

Spinal cord slices were incubated for 2 min at 35 °C with 30 mM KCl in the absence (KCl - PI) or the presence (other bars) of peptidase inhibitors. Control: peptidase inhibitors without 30 mM KCl. KCl + lidocaine: 30 mM KCl, peptidase inhibitors and 1 mM lidocaine. KCl 30 mM was prepared by iso-osmotic replacement of NaCl in aCSF. Peptidase inhibitors and lidocaine were added starting 5 min before KCl. ANOVA revealed a significant effects ($p < 0.0001$). Bonferroni's post test: *** $p < 0.001$ compared with control, ††† $p < 0.001$.

Figure 6. Effect of incubation time on MOR internalization evoked by veratridine and KCl
 Spinal cord slices were incubated for different times at 35 °C with peptidase inhibitors and 50 mM KCl (A) or 30 μ M veratridine (B). KCl 50 mM was prepared by iso-osmotic replacement of NaCl in aCSF. Time 0 min was determined in the absence of veratridine or 50 mM KCl. ANOVA revealed a significant effect of time ($p < 0.0001$). Bonferroni's post test: *** $p < 0.001$ compared with 0 min, ††† $p < 0.001$.

Figure 7. Inhibition of MOR internalization induced by endomorphin-2 by 50 mM KCl for 20 min Spinal cord slices were incubated at 35 °C for 20 min with 1 μ M endomorphin-2 alone (EM-2) or with 50 mM KCl (EM-2 + KCl). KCl 50 mM was prepared by iso-osmotic replacement of NaCl in aCSF. Data points are the mean $\pm$ SEM of 3 slices. *** $p < 0.001$ (t-test).