Arylacetamide κ -opioid Receptor Agonists Produce a Tonic- and Use-dependent Block of Tetrodotoxin-sensitive and -resistant Sodium Currents in Colon Sensory Neurons

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Supported by NS 19912 and NS 35790

Abbreviated title: sodium current blockade by arylacetamide κ -opioids

Number of text pages: 25, figures: 4, references: 28

Number of words in the abstract: 239; introduction: 436; and discussion: 682

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ABSTRACT

We have previously reported that U50,488 enantiomers contribute to visceral antinociception by a non-opioid receptor mediated blockade of sodium currents in colon sensory neurons. The present experiments were undertaken to examine the effect of arylacetamide κ -opioid receptor agonists (κ -ORAs) U50,488 and EMD 61,753 on tetrodotoxin-sensitive (TTX-S) and -resistant (TTX-R) sodium currents, and the mechanism of their sodium channel-blocking actions. Whole cell patch clamp experiments were performed on colon sensory neurons from the S1 dorsal root ganglion identified by content of retrograde tracer Dil. The concentration-response curves of U50,488 and EMD 61,753, for tonic inhibition of total, TTX-S and TTX-R sodium currents were similar (EC_{50} values for U50,488 and EMD 61,753 were $8.4 \pm 1.69 \mu\text{M}$ and $1.2 \pm 1.78 \mu\text{M}$, respectively). In contrast, the peptide κ -ORA dynorphin was without effect in these experiments. U50,488 ($10 \mu\text{M}$) shifted the voltage dependence of steady-state inactivation curves for total, TTX-S and TTX-R currents to more negative potentials. Inhibition was present at holding potentials of -100 to -20 mV. Following the tonic block elicited by $10 \mu\text{M}$ U50,488, repetitive stimulation with 5 ms depolarizing pulses at a frequency of 3 Hz further enhanced the inhibition of total, TTX-R and TTX-S currents by $43.8 \pm 4.9 \%$, $46.2 \pm 4.9 \%$ and $40 \pm 3.2 \%$, respectively. These results demonstrate that arylacetamide κ -ORAs non-selectively inhibit voltage-evoked sodium currents in a manner similar to local anesthetics, by enhancing closed-state inactivation and induction of use-dependent block.

The physiological effects of opioid receptor agonists (ORAs) mediated by their activation of either μ -, δ - or κ -opioid receptors have been well characterized. We previously reported peripheral, visceral antinociceptive actions produced by arylacetamide κ -ORAs like U50,488 and EMD 61,753. These drugs dose-dependently attenuated responses of rat pelvic nerve afferent fibers to noxious distension of the colon or bladder (Su et al., 1997a; Su et al., 1997b; Sengupta et al., 1996). Such peripheral, visceral antinociceptive effects were resistant to antagonism by traditional KOR antagonists like nor-BNI, DIPPA and high-dose naloxone, and were interpreted to be opioid receptor-independent. Further support for this interpretation derives from experiments performed in rats in which the κ -opioid receptor (KOR) was 'knocked-down' at peripheral somatic and colonic sites using antisense oligodeoxynucleotides (Joshi et al., 2000). Although κ -ORA-produced somatic antinociception was blocked by KOR antisense treatment, κ -ORAs could still dose-dependently inhibit responses of pelvic nerve afferent fibers to noxious colon distension in the same KOR antisense-treated rats. Other reports have also described opioid receptor-independent pharmacological actions of κ -ORAs in the cardiovascular (see Pugsley, 2002 for review) and nervous systems (Alzheimer and ten Bruggencate, 1990; Su et al., 2002), including ion channel blocking effects on voltage-gated calcium, potassium and sodium channels.

In particular, κ -ORAs with an arylacetamide structure have been documented in a series of experiments to be antiarrhythmic by a blockade of

cardiac sodium channels (Pugsley et al., 1993; Pugsley et al., 1994; Pugsley et al., 2000a; Pugsley et al., 2000b). The sodium channel blocking action of arylacetamide κ -ORAs persists in the presence of opioid receptor antagonists (Pugsley et al., 1994). When tested in heterologous expression systems without endogenous opioid receptors, the inhibition of sodium currents by such κ -ORAs also persists (Pugsley et al., 2000b). Additionally, the sodium channel blocking action of arylacetamide κ -ORAs is also seen with enantiomers of κ -ORAs that lack activity at opioid receptors (Pugsley et al., 1993). Such observations support the notion that the sodium channel blockade produced by arylacetamide κ -ORAs occurs by a mechanism independent of their actions at the κ -opioid receptor (KOR).

Primary sensory neurons express several voltage-gated sodium channels, some of which are resistant to blockade by tetrodotoxin (TTX) (Black et al., 1996; Bielefeldt, 2000; Elliott and Elliott, 1993; Schild and Kunze, 1997; Bielefeldt et al., 2002). Recent studies suggest that TTX-resistant sodium channels play an important role in nociception, particularly in circumstances where nociceptor excitability is increased after tissue injury (Lai et al., 2002; Bielefeldt et al., 2002). Because κ -ORAs may be useful in management of discomfort and pain that characterize functional gastrointestinal disorders, we examined the effects of κ -ORAs on TTX-sensitive and TTX-resistant sodium currents in colon sensory neurons.

METHODS

Experimental animals and Dil labeling

All experiments were performed on male Sprague Dawley rats (age 2-3 months; Harlan, Indianapolis, IN). Animals were housed 1-2 per cage with free access to food and water, and were maintained on a 12 hr light-dark cycle in the AAALAC approved animal care facility. All experimental protocols were approved by the Institutional Animal Care and Use committee, The University of Iowa.

To label colon sensory neurons, rats were anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally). The descending colon was surgically exposed and the retrograde tracer Dil (1.1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine metanesulfonate; 25 mg/0.5 ml methanol) was injected into 15-20 sites (1-2 μ l/site, 30 μ l total) into the wall of the descending colon using a 30-gauge needle. Each injection site was washed with saline to minimize contamination of the adjacent areas with dye. Animals were allowed to recover for 10-14 d following the surgery to allow dye to be transferred to the cell somas of colon sensory neurons in the S1 dorsal root ganglion (DRG).

Cell Dissociation and Culture

Rats were deeply anesthetized with sodium pentobarbital (50 mg/kg intraperitoneally) and the S1 DRG were quickly removed and transferred into ice-cold culture media. The tissue was minced with a surgical blade and incubated

for 1 h at 37° C in modified L-15 Leibovitz medium containing collagenase (type IA, 1 mg/ml), trypsin (type III, 1 mg/ml) and deoxyribonuclease (type IV, 0.1 mg/ml) (all from Sigma Chemical Co., St. Louis, MO). The enzymatic digestion was terminated by adding soybean trypsin inhibitor (2 mg/ml), 3 mmol/l calcium chloride and bovine serum albumin (1 mg/ml) (all from Sigma). After gentle trituration, the tissue fragments were centrifuged at 800 rpm for 5 min and then re-suspended in modified L-15 medium with 5% rat serum and 2% chick embryo extract (Life Technologies). The cells were plated onto poly-L-lysine coated coverslips (0.1%, Electron Microscopy Sciences, Fort Washington, PA), incubated for 3 hr at 37° C in an incubator saturated with water vapor and 5% CO₂, and studied within 8 h. At that time the nerve cell bodies were round and did not have any processes, reducing the potential for space clamp problems during electrophysiological experiments.

Electrophysiological recordings

Cells attached to coverslips were transferred into a 0.5 ml recording chamber onto a stage of an inverted microscope. Colon sensory neurons were identified by observation of their red orange color in fluorescent light with rhodamine filter (excitation wavelength ~546 nm and barrier filter at 580 nm). Each culture dish usually contained about 2-4 labeled cells among a few hundred unlabelled neurons, and only labeled neurons were selected for study. Sodium currents were recorded using the whole cell patch clamp technique with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA) interfaced with a

personal computer. The patch pipettes were pulled from a thin walled borosilicate glass with tip resistances of 1-3 M Ω after fire polishing. Current recordings were filtered at 2 KHz digitized at 10 KHz using a Digidata 200 interface (Axon Instruments), and the series resistance and whole cell capacitance were compensated > 80% in all experiments. The passive membrane properties were monitored during the course of the experiments and cells were used for analysis only if these properties were stable. Signals were stored on a computer for later analysis. Voltage protocols were generated, the data acquired and analyzed using the software package pCLAMP 6.0 (Axon Instruments). The leak current and residual capacitative transients were digitally subtracted using p/n protocol for leak subtraction with p being the test pulse and n=4. Dynorphin 1-13 and different doses of U50,488 and EMD 61,753 were perfused into the bath to study their effects on sodium currents. All experiments were performed at room temperature (21⁰ C).

Solutions and Chemicals

To isolate sodium currents, the pipette solution contained the following (in mM): 115 cesium chloride, 2.3 calcium chloride, 4.8 magnesium chloride 10 EGTA, 10 HEPES, 4 magnesium adenosine triphosphate and 0.5 sodium guanosine triphosphate. The solution was buffered to pH 7.2 with cesium hydroxide and the osmolality was adjusted to 310 mOsmol/l with sucrose. The composition of the extracellular solution was as follows (in mM): 20 sodium chloride, 70 choline chloride, 40 tetraethyl ammonium chloride, 3 magnesium

chloride, 10 HEPES, 0.1 cadmium chloride and 10 glucose. The solution was buffered at pH 7.3 with tetraethyl ammonium hydroxide and the osmolality was adjusted to 310 mOsmol/l with sucrose. Solutions were prepared with analysis grade chemicals obtained from Sigma.

Drugs

U50,488 (MW as salt 405.8; Research Biochemicals Inc., Natick MA), dynorphin 1-13 (MW 1604; Sigma) and EMD 61,753 (MW as salt: 469.1; a gift from E. Merck, Darmstadt, Germany) were dissolved in distilled water.

Data Analysis

All data are expressed as mean $\pm$ SEM. The data were analyzed using Students' t-test. The concentration of drugs producing a 50% inhibition of peak currents (IC_{50}) was calculated using the program GraphPad Prism (version 2). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Effects of U50,488 and EMD 61,753 on voltage activated sodium currents (VASCs)

In initial control experiments, the peak sodium current did not show significant run down over a time course of 20 min under the experimental conditions chosen (102 ± 2 % control; $n = 6$). To study the effects of arylacetamide κ -ORAs on VASCs, voltage steps of 40 ms duration were applied every 20 s from a resting potential of -70 mV to a test potential of 10 mV. Different concentrations (10^{-9} – 10^{-4} M) of U50,488 or EMD 61,753 were perfused into the bath, peak inward currents were analyzed in the absence and presence of these drugs, and the fractional block of sodium current at each concentration was plotted against the concentration of drug. Fig. 1A shows the concentration-response curves for U50,488 and EMD 61,753 in blocking VASCs from colon sensory S1 DRG neurons. Both drugs produced a concentration-dependent inhibition of peak sodium current. The concentrations of U50,488 and EMD 61,753 causing a half-maximal block of peak sodium current (IC_{50}) were 8.4 ± 1.69 μ M and 1.2 ± 1.78 μ M, respectively. In contrast to the effects of U50,488 and EMD 61,753, the peptide κ -ORA dynorphin 1-13 at a concentration of 10^{-5} M was ineffective in inhibiting VASCs (Fig. 1B).

We separated sodium currents based on their biophysical properties. TTX-sensitive (TTX-S) sodium currents have a voltage of half-inactivation of about -70 mV (Cummins and Waxman, 1997; Bielefeldt et al., 2002) and are

essentially fully inactivated at voltages higher than -40 mV. In contrast TTX-resistant (TTX-R) sodium currents inactivate at more depolarized potentials with a voltage of half-inactivation around -25 mV (Cummins and Waxman, 1997; Bielefeldt et al., 2002). Using a prepulse inactivation protocol with a 750 ms step to -40 mV, we were able to isolate the TTX-R sodium current (Cummins and Waxman, 1997; McLean et al., 1988; Roy and Narahashi, 1992). Digital subtraction of this current from the total sodium current reveals the TTX-S current that has a faster onset of activation and inactivation (Fig. 2A). To test the validity of this approach in colon sensory neurons, we compared the relative contribution of the TTX-R current determined electrophysiologically with results obtained in the presence of 1 μ M TTX (Su et al., 1999). Figure 2B shows the close correlation between both measures. While most cells expressed both TTX-R and TTX-S sodium currents, a predominance of one of the pharmacologically distinct sodium currents was found in about one third of the cells (Fig. 2C). As most of the neurons studied were small with a whole cell capacitance of 30.0 ± 1.1 pF, we were not able to examine the relationship between cell size and sodium current properties. While we relied on the distinction of these current components using electrophysiological rather than pharmacological tools in the experiments described below, we will still refer to the current component as TTX-R and TTX-S throughout the text.

Figure 3 illustrates that U50,488 and EMD 61,753 produce a concentration-dependent inhibition of both TTX-S and TTX-R components of the total sodium current, and were thus non-selective in their blockade.

Effect of U50,488 on the voltage dependence of steady state inactivation of VASCs

To study the effects κ -ORAs on the voltage dependence of inactivation, we chose a concentration of U50,488 (10^{-5} M) that produced a marked block of VASCs. For these experiments, cells were held at conditioning potentials between -110 mV and 10 mV for 750 ms before stepping to a test potential of 10 mV. The peak current amplitude was normalized to maximum current amplitude, and plotted as a function of the conditioning prepulse potential in the absence and presence of 10^{-5} M U50,488 (Fig. 4A). The effect of U50,488 on the inactivation of the TTX-S sodium current was determined by electrophysiologically separating the two fractions from the total sodium current by digitally subtracting the current obtained after a prepulse to -40 mV from the current traces at more negative potentials (see above). Five cells expressed more than 80% TTX-R sodium current and were used to examine the steady state inactivation. The results of the electrophysiologically distinct currents were fit with the Boltzmann equation. Under control conditions, the voltages of half-inactivation were -59.8 ± 2.8 mV ($n = 6$) and -23.5 ± 1.2 mV ($n = 5$) for the TTX-S and TTX-R sodium current, respectively. U50,488 shifted the steady state voltage dependence of inactivation curves for total (Fig. 4A), TTX-S (Fig. 4B) and TTX-R (Fig. 4C) sodium currents to more negative potentials. As shown in figures 4B and 4C, the inactivation curves in the presence of U50,488 exhibited distinct shoulders, not allowing a good fit with the Boltzmann equation.

Use-dependent block of VASCs by U50,488

We also examined the ability of U50,488 (10^{-5} M) to induce a use-dependent block of VASCs. Five minutes after administration of U50,488 or vehicle, repetitive stimulation was applied to the cells with 5 ms depolarizing pulses at a frequency of 3 Hz. The evoked peak currents were normalized to the current during the first depolarization (pulse 1) and plotted as a function of pulse number. The peak currents measured in the presence of U50,488 were normalized to the current during the first depolarization in the presence of the drug to emphasize the amount of use-dependent block, independent of the tonic block produced by U50,488. We studied use-dependent block produced by U50,488 in neurons exhibiting a combination of currents (Fig 5A), only TTX-R current (Fig 5B) and only TTX-S current (Fig 5C) as judged by the previously described biophysical characteristics. Repetitive stimulation enhanced the inhibition following the tonic block of total, TTX-S and TTX-R currents by 43.8 ± 4.9 (n=12), 46.2 ± 4.9 (n=3) and 40 ± 3.2 % (n=6), respectively, by the final pulse, demonstrating the ability of U50,488 to induce a significant use-dependent block of VASCs ($p < 0.05$).

DISCUSSION

The experiments described confirm that arylacetamide κ -ORAs exhibit voltage-gated sodium channel blocking properties in primary colon sensory neurons (Su et al., 2002). The key novel findings relate to the effect of these agents on physiologically distinct sodium currents and their mechanisms of action. Interestingly, the potencies of U50,488 and EMD61,753 on the TTX-R current were not appreciably different than their potencies on the TTX-S component of the current.

Sodium channel blocking actions of arylacetamide κ -ORAs in primary sensory neurons

U50,488 and EMD 61,753 concentration-dependently decreased TTX-S and TTX-R sodium currents. In contrast, the peptide κ -ORA dynorphin 1-13 was without effect in these experiments. The concentrations required to achieve inhibition are consistent with previously published studies examining the effects of arylacetamide κ -ORAs on neuronal (Alzheimer and ten Bruggencate, 1990; Su et al., 2002) or cardiac VASCs (Pugsley et al., 2000b). Previous studies have shown that this inhibition of VASCs by arylacetamide κ -ORAs is at least in part due to a use-dependent block of VASCs (Pugsley et al., 1994; Pugsley et al., 2000a; Su et al., 2002). Additionally, they cause a hyperpolarizing shift in the voltage dependence of inactivation curve of sodium currents (Pugsley et al., 1994; Pugsley et al., 2000a). Such sodium channel blocking mechanisms are

analogous to those reported for local anesthetics like lidocaine, which shares structural similarities with arylacetamide κ -ORAs (Pugsley et al., 2000b).

We examined the mechanisms underlying sodium channel inhibition in primary sensory neurons using one of the arylacetamide κ -ORAs, U50,488. The voltage-dependence of inactivation was shifted by U50,488 to more hyperpolarized potentials. As shown in Fig. 4, significant inactivation occurred at voltages more negative than -80 mV. This became especially prominent in cells primarily expressing TTX-R current, which normally does not inactivate at prepulse potentials negative of -50 mV. These findings are consistent with an enhanced transition from the closed to the inactivated state (Bean et al., 1983) in the presence of the arylacetamide κ -ORA. Comparable findings have been described for cardiac (Pugsley, et al., 1994) and heterologously expressed neuronal sodium channels (Pugsley et al., 2000a). This increased closed state inactivation underlies the tonic inhibition component seen following drug administration.

However, other mechanisms may contribute to the inhibitory effects of arylacetamide κ -ORAs (Pugsley et al., 1994; Pugsley et al., 2000a; Su et al., 2002). We examined whether U50,488 caused a use-dependent block, using a repetitive stimulation protocol. While brief depolarizations to 10 mV at a frequency of 3 Hz did not lead to a cumulative inhibition under control conditions, the peak sodium current progressively decreased in the presence of the κ -ORA. This use-dependent inhibition was similar in both TTX-S and TTX-R VASCs. The presence of use-dependent inhibition, often also referred to as phasic channel

block, suggests that κ -ORAs also interact with the open channel, favoring a transition into the closed state or directly acting on the exposed pore of the ion channels. Local anesthetics lead to similar tonic and phasic inhibition of sodium currents by enhancing transitions into the closed state and slowing reactivation (Courtney, 1975; Wagner et al., 1999).

Peripheral, visceral analgesic actions of arylacetamide κ -ORAs

Previous findings obtained *in vivo* are consistent with a peripheral, opioid receptor-independent mechanism of action for arylacetamide κ -ORAs. Whereas μ -, δ - and peptide κ -ORAs were ineffective when tested for peripheral effects in models of visceral nociception, arylacetamide κ -ORAs exhibited potent antinociceptive effects (Sengupta et al., 1996; Su et al., 1997a; Su et al., 1997b, Ozaki et al., 2000). Opioid receptor antagonists or intrathecal treatment with antisense oligodeoxynucleotides targeting the KOR (Joshi et al., 2000) did not inhibit the peripheral, visceral antinociceptive actions of arylacetamide κ -ORAs. Arylacetamide κ -ORAs, including enantiomers of U50,488 with no efficacy as κ -ORAs in KOR antisense-treated animals produce, are antinociceptive at peripheral cutaneous sites like the hind paw, but only at doses higher than those required to activate the KOR (Joshi and Gebhart, in press).

Significance of findings

Sodium channels play a significant role in the perception of noxious sensation arising from the viscera (Bielefeldt et al., 2002; Gebhart et al., 2002;

Yoshimura et al., 2001). Enhanced activation of sodium channels is thought to underlie the activation of primary afferent nerves after inflammation of bladder, colon and stomach by chemical irritants (Bielefeldt et al., 2002; Beyak et al., 2003). The sodium channel blocking actions of κ -ORAs in primary sensory neurons therefore have important implications. Results obtained *in vivo* demonstrate that such actions contribute to the peripheral analgesia produced by κ -ORAs. Such drugs may be clinically useful also in situations such as neuropathic pain, when changes in sodium channel expression lead to increases in action potential generation (reviewed in Waxman et al., 1999). The use-dependent (phasic) inhibition may preferentially affect cells with abnormal action potential discharge, thus potentially decreasing the likelihood of adverse effects. Similar mechanisms of actions have been proposed for other use-dependent sodium channel blockers like mexiletine, which have been used for the treatment of neuropathic pain (Tanelian and Brose, 1991; Wagner et al., 1999).

ACKNOWLEDGEMENTS

The authors thank Mike Burcham for the production of graphics and Sherry Kardos for help with cell culture.

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FIGURE LEGENDS

Figure 1. Effects of κ -opioid receptor agonists on voltage-activated sodium currents in colon sensory neurons. Voltage steps of 40 ms duration were applied every 20 sec from a resting potential of -70 mV to a test potential of 10 mV, and currents were recorded in the presence and absence of the drugs. A, U50,488 and EMD 61,753 produced concentration-dependent inhibition of total sodium currents in S1 colon sensory neurons. IC_{50} values for U50,488 and EMD 61,753 were 8.4 ± 1.69 μ M and 1.2 ± 1.78 μ M, respectively. B, The peptide κ -ORA dynorphin 1-13 did not inhibit voltage-activated sodium currents. Representative traces shown in the inset demonstrate the effect of the drugs on total sodium current. The inhibition produced by U50,488 and EMD 61,753 was reversible upon washing (recovery).

Figure 2. Separation of distinct voltage-sensitive sodium currents. A, Electrophysiological separation of TTX-S and TTX-R components of the total sodium current. Total sodium current was obtained using a 750 ms prepulse to -110 mV before applying the test pulse to 10 mV. Using a prepulse inactivation protocol with a 750 ms step to -40 mV, the TTX-R sodium current was isolated. Digital subtraction of this current from the total sodium current reveals the TTX-S current that has a faster onset of activation and inactivation. B, Results of the electrophysiological isolation of TTX-R sodium currents with a prepulse to -40 mV (expressed as fraction of the total sodium current) significantly correlated

with the effects of 1 μ M TTX (from Su et al., 1999, expressed as fraction of the total sodium current; n = 24). C, The relative contribution of TTX-S sodium current to the total sodium current was examined using 1 μ M TTX, demonstrating a mixed current in most cells.

Figure 3. Effect of κ -opioid receptor agonists on TTX-S and TTX-R components of the total sodium current. U50,488 and EMD 61,753 non-selectively produced a tonic blockade of both TTX-S (A) and TTX-R (B) components of the total sodium current.

Figure 4. Effect of U50,488 on voltage dependence of steady state inactivation of sodium current. A two-pulse protocol (see text for details) was used in these experiments. The peak current amplitude evoked by the test pulse was normalized to the maximum current amplitude and plotted as a function of the conditioning prepulse potential in the absence (o) and presence (•) of drug. The voltage of half-inactivation was determined under control conditions for the electrophysiologically distinct currents (TTX-S -58.3 mV; TTX-R -22.2 mV). U50,488 (10^{-5} M) shifted the inactivation curves for total (A), TTX-S (B) and TTX-R (C) to more negative potentials.

Figure 5. Use-dependent block of sodium currents by U50,488. The ability of U50,488 (10^{-5} M) to induce a phasic block was studied in cells expressing both

(A), only TTX-S (B) and TTX-R (C) sodium currents. Repetitive stimulation with 5 ms depolarizing pulses from a resting potential of -70 mV to a test potential of 10 mV at a frequency of 3 Hz was given in the presence and absence of drug. The peak currents were normalized to the currents during the first depolarization (pulse 1) and plotted as a function of pulse number. The peak currents plotted in the presence of the drug were normalized to the current during the first depolarization in the presence of the drug. A progressive decrease in currents with repetitive stimulation is observed following perfusion of U50,488. The ratio of current amplitude at the final pulse decreased significantly to $\sim 40\%$ for the total, TTX-S and TTX-R sodium currents ($p < 0.05$).

Key Words: kappa-opioid receptor agonist, sodium channel blocker, local anesthetic, colon sensory neuron, arylacetamide, whole cell patch clamp