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RESEARCH PAPER

Activation of μ-opioid receptors and block of $K_{IR}3$ potassium channels and NMDA receptor conductance by *l*- and *d*-methadone in rat locus coeruleus

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BACKGROUND AND PURPOSE

Methadone activates opioid receptors to increase a potassium conductance mediated by G-protein-coupled, inwardly rectifying, potassium ($K_{IR}3$) channels. Methadone also blocks $K_{IR}3$ channels and N-methyl-D-aspartic acid (NMDA) receptors. However, the concentration dependence and stereospecificity of receptor activation and channel blockade by methadone on single neurons has not been characterized.

EXPERIMENTAL APPROACH

Intracellular and whole-cell recording were made from locus coeruleus neurons in brain slices and the activation of μ-opioid receptors and blockade of $K_{IR}3$ and NMDA channels with *l*- and *d*-methadone was examined.

KEY RESULTS

The potency of *l*-methadone, measured by the amplitude of hyperpolarization was 16.5-fold higher than with *d*-methadone. A maximum hyperpolarization was caused by both enantiomers (~30 mV); however, the maximum outward current measured with whole-cell voltage-clamp recording was smaller than the current induced by [Met]^{5}enkephalin. The $K_{IR}3$ conductance induced by activation of α_2-adrenoceptors was decreased with high concentrations of *l*- and *d*-methadone (10–30 μM). In addition, methadone blocked the resting inward rectifying conductance (K_{IR}). Both *l*- and *d*-methadone blocked the NMDA receptor-dependent current. The block of NMDA receptor-dependent current was voltage-dependent suggesting that methadone acted as a channel blocker.

CONCLUSIONS AND IMPLICATIONS

Methadone activated μ-opioid receptors at low concentrations in a stereospecific manner. $K_{IR}3$ and NMDA receptor channel block was not stereospecific and required substantially higher concentrations. The separation in the concentration range suggests that the activation of μ-opioid receptors rather than the channel blocking properties mediate both the therapeutic and toxic actions of methadone.

Abbreviations

ACSF, artificial cerebrospinal fluid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; β-CNA, β-chlornaltrexamine; K_{IR}, inward rectifying channel; $K_{IR}3$, G-protein-coupled, inwardly rectifying, potassium channel; LC, locus coeruleus; ME, [Met]^{5}enkephalin; NBQX, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione; NMDA, N-methyl-D-aspartic acid

Introduction

Methadone is used commonly for the treatment of opiate dependence (Dole and Nyswander, 1965). The initiation of treatment even in tolerant individuals begins with low doses that are increased over time until a high degree of tolerance to opioids is attained. With the increase in dose, non-opioid receptor-dependent actions of methadone may account for some of the therapeutic and/or toxic consequences (Kreek, 2000). In addition to the activation of opioid receptors, methadone blocks ion channels, such as G-protein-coupled inwardly rectifying potassium ($K_{IR}3$) channels and N-methyl-D-aspartic acid (NMDA) receptors (Gorman *et al.*, 1997; Ebert *et al.*, 1998; Rodriguez-Martin *et al.*, 2008; receptor and channel nomenclature follows Alexander *et al.*, 2009). The block of NMDA receptors by methadone has been identified as a potentially important therapeutic action because the NMDA receptor antagonist, MK801, reduced hyperalgesia (Mao *et al.*, 1992), enhanced opiate analgesia (Ebert *et al.*, 1998) and attenuated morphine tolerance and dependence (Trujillo and Akil, 1991; Elliott *et al.*, 1995; Bilsky *et al.*, 1996). It is possible that in individuals receiving the highest doses of methadone, the channel blocking actions may contribute to the therapeutic outcome. It is therefore necessary to determine the concentrations of methadone that are required to activate opioid receptors relative to the concentrations that result in channel block.

Stereoselective binding of ligands to opioid receptors was one of the properties used to define receptor-specific binding (Pert and Snyder, 1973a,b). The enantiomers of methadone differentially activate μ-opioid receptors. Depending on the assay, *l*-methadone was 4–50 times more potent than *d*-methadone (Scott *et al.*, 1948; Wallisch *et al.*, 2007). Thus, like enantiomers of morphine, naloxone and many other natural opiates, the *l*-methadone binds to μ-opioid receptors selectively over the *d*-enantiomer (Pert and Snyder, 1973a,b). The present study examines the potency and efficacy of the two enantiomers and racemic (*l*/*d*) methadone to activate μ-opioid receptors and block ion channels ($K_{IR}3$ channel and NMDA receptor-dependent currents). Recordings were made from locus coeruleus (LC) neurons in brain slices and the concentration dependence and stereoselectivity of the activation of μ-opioid receptors was compared with the blockade of $K_{IR}3$ channel and NMDA receptor-dependent currents.

The results show that *l*-methadone was 16.5-fold more potent than *d*-methadone in the activation of $K_{IR}3$ channel conductance. At higher concentrations, both enantiomers of methadone reduced the $K_{IR}3$ channel conductance stimulated by activation of α_2-adrenoceptors, as well as the NMDA receptor-dependent current. The results suggest that therapeutic concentrations of methadone do not reach a concentration that is sufficient to block $K_{IR}3$ channels and NMDA receptors. Thus the actions of methadone are primarily dependent on the activation of μ-opioid receptors.

Methods

Preparation of brain slices and solutions

All animal care and experimental procedures were in accordance with the National Institutes of Health guidelines and approved by the Institutional Animal Care and Use Committee at Oregon Health and Science University. Male Sprague–Dawley rats (150–300 g) were obtained from Charles River. The details of the methods for slice preparation and recording have been reported elsewhere (Williams *et al.*, 1984). Briefly, the rats were anaesthetized with isoflurane and killed. The brain was removed and placed in a vibratome (Leica) containing cold (4°C) oxygenated (95% O_2, 5% CO_2) artificial cerebrospinal fluid (ACSF) containing (in mM) 126 NaCl, 2.5 KCl, 1.2 $MgCl_2$, 2.4 $CaCl_2$, 1.2 NaH_2PO_4, 25 $NaHCO_3$ and 11 D-glucose. In experiments where the NMDA receptor-dependent current was examined, slices were cut and incubated in kynurinate (1.5 mM) until being placed in the recording chamber, otherwise the cutting and incubation solution contained MK801 (3 μM). Horizontal slices (220–230 and 280–300 μm for whole-cell and intercellular recording respectively) containing the LC were obtained from the rostral pons. Slices were incubated for at least 30 min before recording in warm (35°C) oxygenated ACSF that contained MK801 (10 μM), or kynurinate (1.5 mM) in experiments where the current induced by NMDA receptor activation was examined.

Recordings

Slices were placed in a chamber and superfused with ACSF (35°C), at 1.5 mL·min^{-1}. In experiments that used intracellular recording, the area of the LC was visualized using a dissecting microscope located just lateral to the fourth ventricle and recordings were made blind. Intracellular electrodes (50–100 MΩ) were filled with 2 M KCl. LC neurons were identified by their intrinsic electric properties including, spontaneous firing at a rate of 1–5 Hz, an action potential that was 80 mV, with a threshold of –55 mV, a duration of 1.2–1.4 ms and a large afterhyperpolarization. Hyperpolarizing current (5–15 pA) was applied to maintain the membrane potential just below

the threshold for action potential generation (≈–60 mV). Data were collected using Power Lab (Chart software; ADInstruments, Colorado Springs, CO) and acquired at 200 Hz.

Whole-cell voltage-clamp recordings were made with a 40× water-immersion objective on an upright microscope (Zeiss Axioscop 1) equipped with gradient contrast infrared optics. Glass pipettes contained (in mM): 126 K methylsulphate (or 115 CsCl), 20 NaCl, 10 HEPES, 10 BAPTA, 2 Mg ATP, 0.2 Na_2 GTP, and 10 Na_2 phosphocreatine, at pH 7.3, and 275 mOsm. Pipette resistance was 1.5–3 MΩ. Acceptable access resistance was <15 MΩ. Series resistance compensation was not applied. Slices were placed in the recording chamber and superfused with oxygenated (95% O_2, 5% CO_2) ACSF at 35°C. An Axopatch 1D amplifier (Molecular Devices) was filtered at 5 kHz, digitized at 10 kHz using AxoGraph X (AxoGraphX) and stored on a Macintosh computer.

Iontophoretic pipettes were pulled with the resistance of ≈100 MΩ, and filled with L-aspartate (1 M) or noradrenaline (500 mM). The iontophoretic pipette was placed adjacent to the soma within 5–20 µm. A backing current (≈1 nA) was applied. L-aspartate was ejected with negative current (≈50 nA) for 20 ms once every 20 s using an Axoclamp 2A amplifier (Molecular Devices). Noradrenaline was applied with positive current (50 nA) once every 30 s. The current evoked by NMDA receptors was pharmacologically isolated using the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor antagonist, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX; 5 µM).

Data analysis

Dose–response curves were constructed by normalizing the amplitude of the hyperpolarization induced by *l*- or *d*-methadone to the hyperpolarization induced by a saturating concentration of the α_2-adrenoceptor agonist, UK14304 (3 µM). A single recording was made in each slice and only one concentration of methadone was applied. The duration of the application varied with concentration so that a steady-state hyperpolarization was obtained (up to 60 min). Once a steady-state hyperpolarization was obtained, naloxone (1 µM) was applied to reverse the hyperpolarization. Then, UK14304 (3 µM), was applied to activate the same population of $K_{IR}3$ channels and reverse its effect with the antagonist, yohimbine (2 µM). The hyperpolarization induced by methadone was then normalized to that induced by UK14304, to eliminate cell-to-cell variability. This protocol was necessary because the time course of the hyperpolarization induced by methadone, particularly *d*-methadone was very slow and the most accurate measure of the amplitude of the hyperpolarization was the difference in membrane potential before and after the addition of naloxone. In experiments where the block NMDA receptors were examined methadone was added cumulatively. The maximal hyperpolarization (E_{max}), EC_{50} and IC_{50} values were determined using KaleidaGraph software (Synergy Software) calculated using a logistic function.

Data were expressed as mean ± SEM. Statistical significance was determined with unpaired Student's *t*-test or one-way ANOVA followed by the *post hoc* Student Newman–Keuls test. $P \leq 0.05$ was considered to show significance.

Materials

Drugs were applied by bath perfusion. The solution containing [Met]^{5}enkephalin (ME) also included the peptidase inhibitors, bestatin hydrochloride (10 µM) and thiorphan (1 µM). Racemic methadone, *l*- and *d*-methadone, morphine sulphate and cocaine were obtained from NIDA (National Institute on Drug Abuse, Bethesda, MD, USA). UK14304, kynuric acid and β-chlornaltrexamine (β-CNA) were from Tocris Cookson (Ellisville, MO, USA). All other drugs were from Sigma (St Louis, MO, USA). MK801, noradrenaline, UK14304, thiorphan, β-CNA and NBQX were dissolved in dimethyl sulphoxide. The final concentration of dimethyl sulphoxide did not exceed 0.01%. All other drugs were dissolved in water.

Results

l- *and* d-*methadone hyperpolarize LC neurons*

The activation of µ-opioid receptors by *l*- and *d*-methadone was first examined by application of a single concentration of each enantiomer (1 µM). Both *l*- and *d*-methadone caused a hyperpolarization that was reversed by naloxone (1 µM, Figure 1). The amplitude of the hyperpolarization induced by *l*-methadone was 26.0 ± 2.3 mV (n = 5), whereas *d*-methadone resulted in a hyperpolarization of 17.7 ± 1.7 mV (n = 8; Figure 1A, P = 0.01). The onset of the hyperpolarization induced by *l*- and *d*-methadone (1 µM) also differed. The time it took to reach the half amplitude of the steady hyperpolarization was 5.4 ± 0.9 min (n = 5) for *l*-methadone and 22.9 ± 2.7 min (n = 7) for *d*-methadone (P = 0.002). Thus, *l*-methadone (1 µM) was more potent than *d*-methadone (1 µM). As a control for the long duration application of methadone, a saturating concentration of UK14304 (3 µM) was applied at the end of each experiment. The amplitude of the

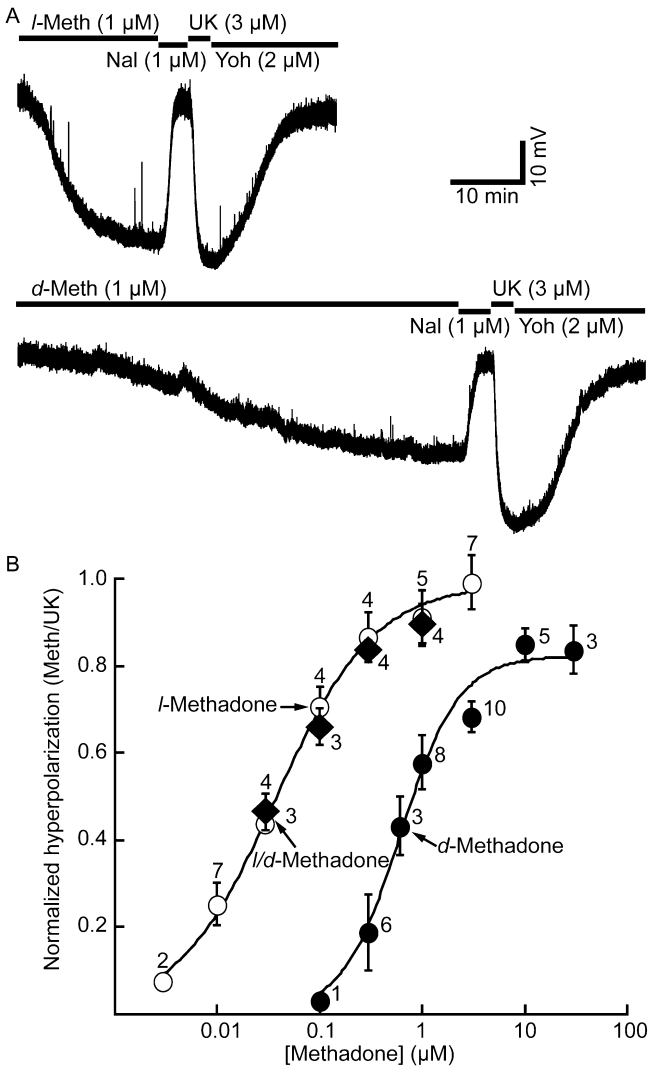

Figure 1

The concentration response curves for *l*- and *d*-methadone are significantly different. A, representative traces of the hyperpolarization induced by *l*-methadone (*l*-Meth; 1 μM; top) and *d*-methadone (*d*-Meth; 1 μM; bottom). In each case the hyperpolarization was reversed with naloxone (Nal; 1 μM). The hyperpolarization induced by UK14304 (UK; 3 μM) was reversed by yohimbine (Yoh; 2 μM). Note the difference in amplitude and the time course of the hyperpolarization induced by *l*- and *d*-methadone (1 μM). B, concentration response curves to *l*-methadone, *d*-methadone and *l*/*d*-methadone. The amplitude of the hyperpolarization was normalized to hyperpolarization induced by UK14304 (3 μM). Error bars indicate ±SEM.

hyperpolarization induced by UK14304 following *l*-methadone (1 μM) was 28.6 ± 1.6 mV ($n = 5$), and after *d*-methadone (1 μM) was 30.5 ± 1.2 mV ($n = 8$, $P > 0.05$). The amplitude of the hyperpolarization was not changed following the prolonged application of methadone (1 μM, 30–60 min).

Dose–response curves to *l*-, *d*- and racemic (*l*/*d*) methadone were constructed by normalizing the hyperpolarization induced by methadone to the hyperpolarization induced by UK14304 (3 μM, Figure 1B). The half maximal effective concentration (EC_{50}) of *l*- and *d*-methadone was 37 ± 3.8 nM and 609 ± 73.4 nM respectively (Figure 1B). When *l*- and *d*-methadone were compared at equally effective concentrations (EC_{50}) there was no difference in the half time it took to reach a steady-state hyperpolarization (*l*-methadone 23 ± 0.8 min, *d*-methadone 23 ± 0.9 min; $n = 3$ each). Thus, *l*-methadone was 16.5-fold more potent than *d*-methadone, and the time course of activation at equally effective concentrations was the same for both enantiomers. The concentration response to *l*/*d*-methadone was not different from *l*-methadone (Figure 1B). The hyperpolarization induced by a maximal concentration of *l*-methadone (3 μM) was 25.4 ± 2.1 mV ($n = 7$), which was not different from that induced by *d*-methadone (10 and 30 μM; 23.7 ± 1.1 mV; $n = 8$; $P > 0.05$). With this assay, both *l*- and *d*-methadone caused a maximal hyperpolarization and by this measure would be considered full opioid agonists.

A second assay was used to address the efficacy of *l*- and *d*-methadone. The outward current induced by a maximal concentration of each isomer was examined using whole-cell voltage-clamp recordings. The use of voltage-clamp is more accurate at estimating the maximal action of opioid agonists because it removes the change in driving force for potassium that results when experiments are carried out under current-clamp conditions. Saturating concentrations of ME (30 μM), morphine (15 μM), *l*-methadone (10 μM) and *d*-methadone (10 μM) were applied, the peak outward currents were measured and normalized to the current induced by UK14304 (3 μM; Figure 2A). The ME (30 μM)-induced current was 123 ± 4.7% of the UK14304 current, whereas morphine (15 μM) caused a current that was only 47.4 ± 7.3% of the UK14304 current. The currents induced by *l*- and *d*-methadone (10 μM) were 56.5 ± 4.6%, and 36.3 ± 2.7% of the UK14304 current respectively (Figure 2B). These observations suggest that both *l*- and *d*-methadone are partial agonists; however, this interpretation may be flawed because the peak current induced by methadone may be blunted by two mechanisms. First, the slow rise in current may result in the combination of the activation of the $K_{IR}3$ channel conductance and the simultaneous development of acute receptor desensitization. Second, methadone is known to block $K_{IR}3$ channel-mediated potassium conductance such that at the high concentrations used to reach the peak the potassium conductance may be reduced.

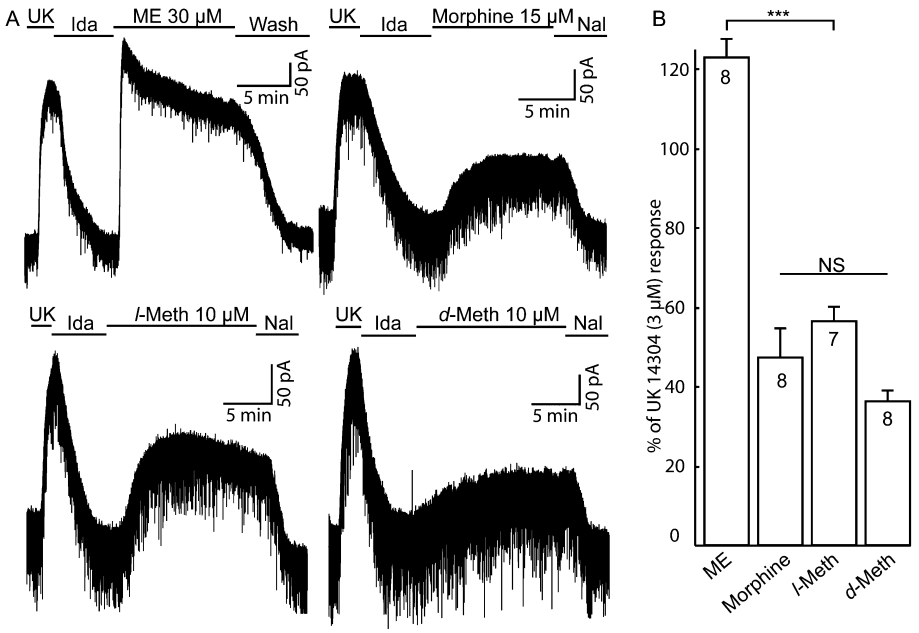

Figure 2

Morphine, *l*- and *d*-methadone are partial agonists. A, raw current traces from whole-cell voltage-clamp recordings. Saturating concentrations of *l*- and *d*-methadone (*l*-, *d*-Meth; 10 μM) and morphine (15 μM) produced outward currents that were less than the current induced by UK14304 (3 μM). The current induced by [Met]^{5}enkephalin (ME) (30 μM) was larger than the current induced by UK14304. B, summarized result showing the current activated by ME (30 μM), morphine (15 μM), *l*- and *d*-methadone (10 μM) normalized to the current induced by UK 14304 (3 μM). The ME current was 123 ± 4.6% of UK14304 current, whereas, current evoked by morphine was 47 ± 7.3% and *l*- and *d*-methadone was 56.5 ± 3.62% and 36.3 ± 2.70%. ***$P < 0.001$, significantly less than the ME current. NS, not significant.

$K_{IR}3$ channel blockade by l- *and* d-*methadone*

The activation of μ-opioid receptors and α_2-adrenoceptors results in an increase in the same $K_{IR}3$ channel conductance (North and Williams, 1985). The hyperpolarization induced by noradrenaline acting on α_2-adrenoceptors was used to examine the blockade of $K_{IR}3$ conductance by methadone. Prior to the experiment, brain slices were incubated with the irreversible opioid receptor antagonist, β-CNA (1 μM, 30 min) to block μ-opioid receptors so that methadone did not cause a hyperpolarization. Noradrenaline (10 μM + cocaine 10 μM) was applied for 2.5 min at 10 min intervals over a period of 1 h. The amplitude of the hyperpolarization caused by noradrenaline was 20.9 ± 0.4 mV ($n = 21$) in control and was stable for 1–2 h. Application of high concentrations of *l*/*d*-methadone (10–30 μM) resulted in a gradual decline in the noradrenaline-induced hyperpolarization over 50 min from 20.9 mV to 12.9 ± 1.1 mV ($n = 4$, Figure 3A). A similar reduction in the hyperpolarization induced by noradrenaline was observed with both *l*- and *d*-methadone (10 μM, *l*-methadone 69 ± 2.1% of control, $n = 7$; *d*-methadone 71 ± 3.3% of control, $n = 8$). The decrease was larger with the application of a higher concentration of methadone (*l*-methadone 30 μM, 54 ± 6.8% of control, $n = 5$; *d*-methadone 47 ± 2.0% of control, $n = 6$, Figure 3C).

The slow time course over which even high concentrations of methadone blocked $K_{IR}3$ channels limited experiments aimed at characterizing the concentration dependence of this action. Therefore, slices were incubated in *d*/*l*-methadone (1–30 μM, naloxone 1 μM, prazosin 100 nM) for 4–8 h prior to making recordings. Following the incubation period and in the continued presence of the appropriate concentration of methadone, whole-cell recordings were made and the current induced by a saturating concentration of noradrenaline (90 μM, cocaine 10 μM, prazosin 100 nM) was applied. In order to reduce cell-to-cell variability, the current induced by noradrenaline was normalized to the capacitance of each cell (Figure 3B). The results indicate that there was a significant decrease in $K_{IR}3$ channel conductance after an incubation period of 4–8 h methadone (10 μM, $P = 0.02$; 30 μM, $P < 0.001$). Incubation in methadone (3 μM) did not result in a significant reduction in the noradrenaline current density ($P = 0.2$). Thus, methadone partially blocked $K_{IR}3$ conductance in dose-dependent manner that was not stereoselective.

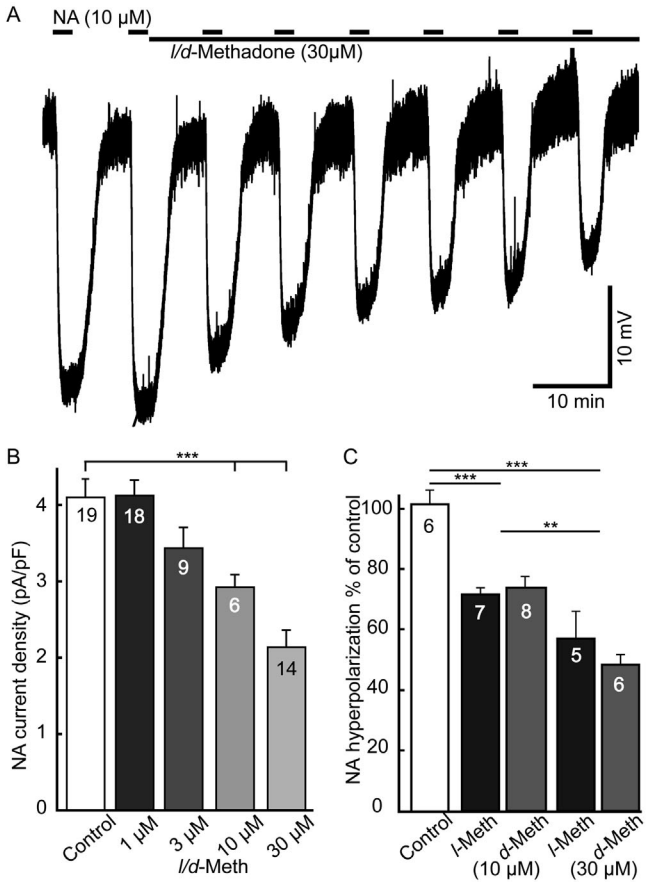

Figure 3

Both *l*- and *d*-methadone decreased the hyperpolarization induced by noradrenaline (NA). A, representative intracellular recording showing the protocol used. The hyperpolarization induced by noradrenaline (NA 10 μM + cocaine 10 μM) was reduced from 20.9 mV to 12.0 mV by both *l*/*d*-methadone (30 μM) over a period of 1 h. Slices were treated with β-chlornaltrexamine (β-CNA) prior to the experiments, so that methadone did not induce a hyperpolarization. B, summary of the concentration-dependent inhibition of the current density (pA/pF) induced by noradrenaline (90 μM + cocaine 10 μM). Slices were incubated for 4–8 h in the indicated concentration of methadone prior to the experiment. There was a significant decrease in noradrenaline-induced current density in slices treated with 10 and 30 μM methadone. C, summary of concentration-dependent block of noradrenaline-induced hyperpolarization measured after 40–50 min. There was no difference in the reduction of hyperpolarization between *l*- and *d*-methadone. Error bars indicate ±SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$, different from control.

When current/voltage plots were constructed in slices that were incubated in methadone (10 μM, and naloxone, 1 μM, >1 h), there was a dramatic decrease in the conductance mediated by the inward rectifying potassium conductance (K_{IR}, Figure 4C). The voltage dependence of the block of $K_{IR}3$ channels by *d*/*l*-methadone was examined in experiments where I/V curves were constructed with a ramp protocol (–55 to –135 mV, 0.5 s) before and after noradrenaline was applied by iontophoresis (Figure 4A). This protocol was repeated at 30 s intervals in the continued presence of *d*/*l*-methadone (1 and 10 μM, naloxone 1 μM). There was no change in the noradrenaline I/V plots induced by methadone (1 μM, 15 min) whereas methadone (10 μM, 10 min) reduced the noradrenaline-induced current over the entire voltage range (Figure 4B,D).

Methadone blocks NMDA receptor-mediated current

Previous studies have shown that methadone inhibits NMDA receptor current by binding at the same site as MK801 (Gorman *et al.*, 1997; Ebert *et al.*, 1998). Whole-cell voltage-clamp recordings were used to examine the actions of methadone on the current induced by the activation of NMDA receptors. Slices were treated with β-CNA (1 μM, 30 min) prior to recording in order to block μ-opioid receptors. Extracellular magnesium was removed in order to measure NMDA receptor-dependent currents induced while holding the cell at –60 mV. Iontophoretic application of L-aspartate (50 nA, 20 ms) was used to activate NMDA receptors every 20 s in the presence of the AMPA receptor antagonist NBQX (5 μM). The current induced by L-aspartate was completely blocked by the non-selective glutamate antagonist, kynurenic acid (1.5 mM; data not shown). After steady NMDA-induced currents were obtained for at least 5 min, *d*-, *l*- or *l*/*d*-methadone were superfused. The amplitude of the NMDA current progressively declined following the addition of methadone and reached a steady state within 15–20 min. The extent of the inhibition was dependent on the concentration of *l*/*d*-methadone applied (Figure 5C). The inhibition curve indicated that the IC_{50} value for *l*/*d*-methadone was 3.5 ± 0.3 μM (Figure 5C). At 3 and 30 μM both enantiomers of methadone decreased the NMDA-induced current equally (*l*-methadone 3 μM 52 ± 6% of control, $n = 7$, 30 μM 16 ± 2% of control, $n = 6$; *d*-methadone 3 μM 59 ± 6% of control, $n = 8$, 30 μM 21 ± 2% of control, $n = 7$, Figure 5B). Therefore, the block of NMDA receptor-dependent current was not stereoselective.

The voltage dependence of the block of NMDA-induced current by *l*/*d*-methadone was examined to determine whether the inhibition resulted from a channel block. The amplitude of the current induced by NMDA receptors was examined over a range of –100 to +40 mV in 20 mV increments. In each experiment, the amplitude of NMDA-induced current was normalized to the current measured at –60 mV in control and I/V plots were constructed (Figure 5D). When LC neurons were held at –60 mV, *l*/*d*-methadone (30 μM) decreased the NMDA-induced current to 33 ± 6.4% ($n = 4$, $P < 0.0001$) of

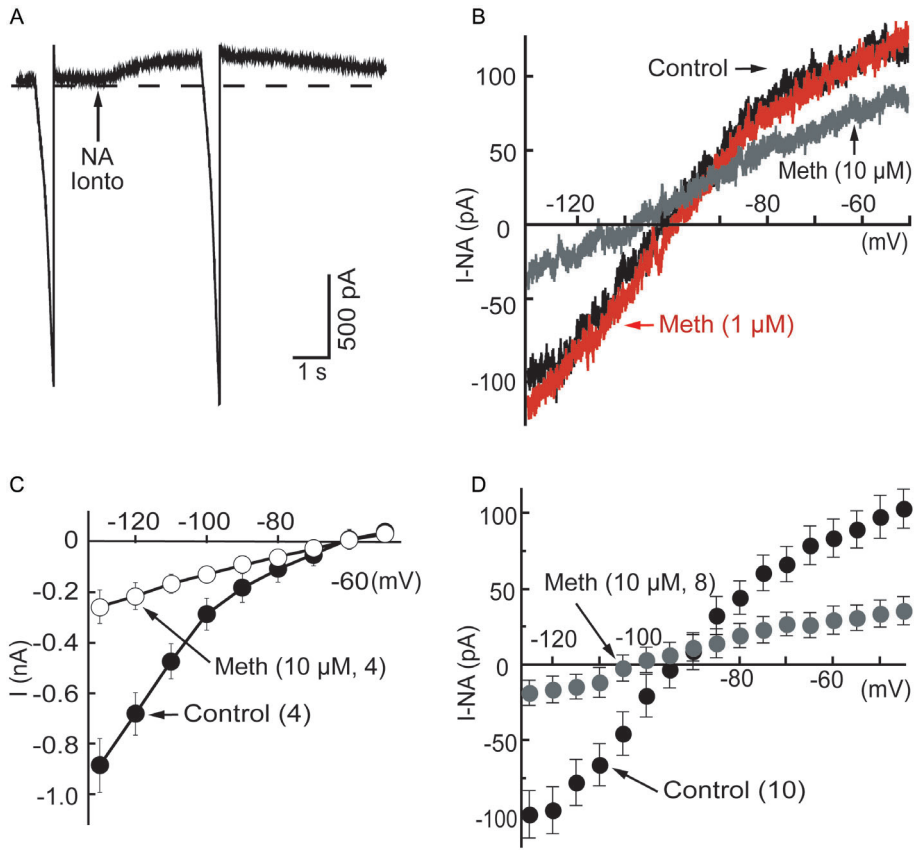

Figure 4

Voltage dependence of inward rectifying channel (K_{IR}) block by methadone. A, raw current trace illustrating the protocol used to characterize the voltage dependence of the current induced by the iontophoretic application of noradrenaline (NA; arrow). B, recording from a single neuron showing the current/voltage plot in control (control, black), after superfusion of 1 μM methadone (Meth 1 μM, red) and 10 μM (Meth 10 μM, gray). C, methadone decreased resting conductance. Whole-cell current/voltage plot in the absence (control) and presence of methadone (Meth; 10 μM, $n = 4$ each). D, summarized current/voltage illustrating the current induced by noradrenaline in control and after methadone (10 μM). Opioid receptors were blocked in all experiments with either β-chlornaltrexamine or naloxone.

control. At +40 mV however, the amplitude of the NMDA current was not significantly different from control (63 ± 14% of the control current at –60 mV) and methadone (61 ± 19% of the control current at –60 mV). Thus, methadone blocked the inward current induced by the activation of NMDA receptors but had no effect on outward current measured at positive membrane potentials. This result is consistent with methadone acting as a pore blocker of the NMDA receptor.

Discussion and conclusions

A racemic mixture of methadone is used clinically for opiate maintenance therapy primarily because of its prolonged pharmacokinetics (Dole and Nyswander, 1965). In addition to the action on opioid receptors, blockade of NMDA receptors is thought to facilitate the treatment of opiate addiction (Ebert *et al.*, 1998). This study demonstrated that *l*-methadone was more potent and efficacious than *d*-methadone on μ-opioid receptors measured by activation of potassium conductance. The results indicate that the concentration of methadone required to activate μ-opioid receptors was significantly lower than that required to block $K_{IR}3$ channels and NMDA-induced conductance and the channel blocking action was not stereoselective. Thus the action of methadone when used at therapeutic levels is primarily mediated by opioid receptors and channel blocking actions may only be observed after reaching very high levels.

Potency and efficacy of l- *and* d-*methadone*

A distinct difference in potency between *l*- and *d*-methadone was demonstrated in this study. *l*-methadone was 16.5-fold more potent than *d*-methadone at inducing hyperpolarization of LC neurons. Similar results have been reported in the

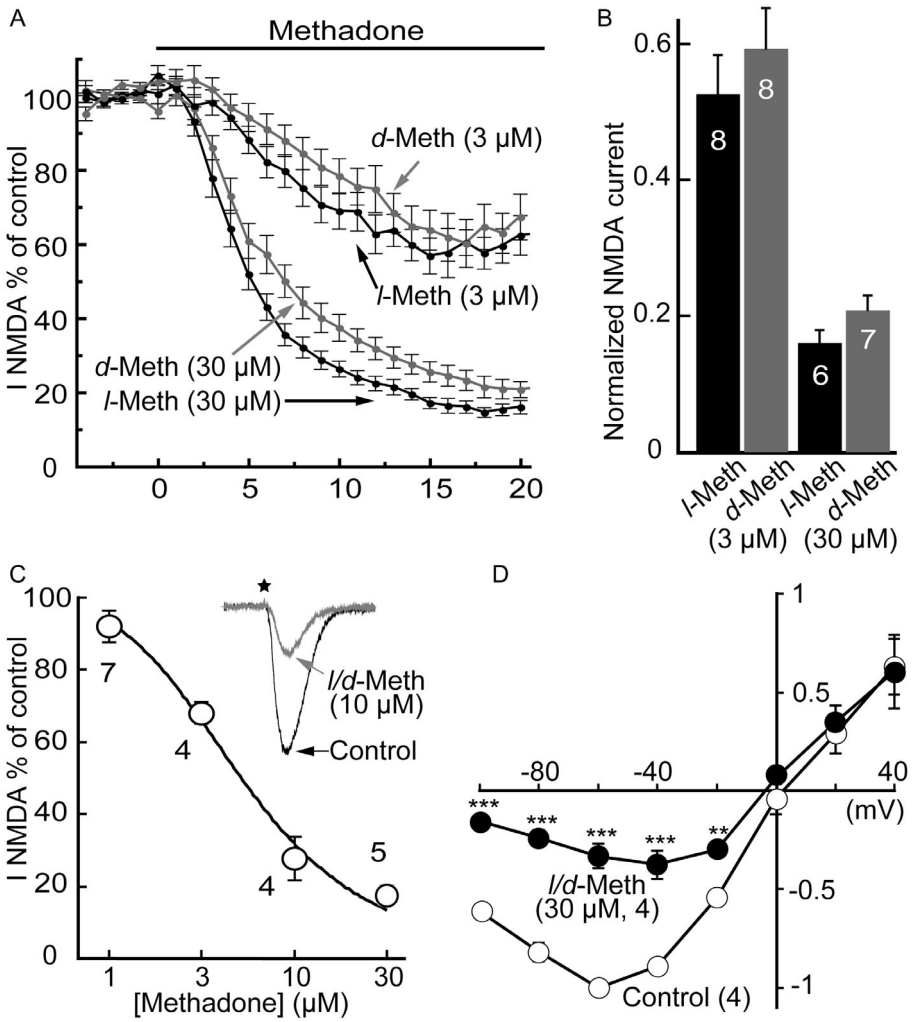

Figure 5

Methadone block of N-methyl-D-aspartic acid (NMDA) receptors. A, summary of the time course of inhibition by *l*- and *d*-methadone (Meth; 3 and 30 μM). B, summary of inhibition of NMDA current after application of *l*- and *d*-methadone (3 and 30 μM). C, the concentration–response curve for the *l*/*d*-methadone block of NMDA-induced currents. Inset are superimposed traces of the inward current induced by iontophoretic application of aspartate in control and after superfusion with methadone (10 μM). D, the current-voltage relationship of NMDA receptor-dependent current in control (open circle) and after application of *l*/*d*-methadone (30 μM; closed circle). The amplitude of NMDA-induced current was normalized to the current measured at −60 mV in control. At negative potentials, NMDA-induced currents were significantly decreased but were not affected at positive potentials. Error bars indicate ±SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$, different from control. Opioid receptors were blocked in all experiments with either β-chlornaltrexamine or naloxone.

binding affinity to μ-opioid receptors and GTPγS binding assay (Codd *et al.*, 1995; Wallisch *et al.*, 2007). The binding affinity of *l*-methadone was 21- to 30-fold higher than *d*-methadone (Codd *et al.*, 1995; Wallisch *et al.*, 2007). In a GTPγS binding assay, *l*-methadone had a 23-fold greater potency than *d*-methadone (Wallisch *et al.*, 2007). The difference in potency was slightly lower in our study (16.5-fold) perhaps because of the difference in the time it took to reach a steady-state hyperpolarization for the two isomers. It required about 1 h to reach a steady-state hyperpolarization with *d*-methadone (1 μM) while a steady state was reached within 15–20 min with *l*-methadone (1 μM). The long duration required for *d*-methadone to reach steady state may have resulted in an accumulation of this lipophilic agonist in slice preparations and thus been an underestimation of the true concentration.

The efficacy of *l*- and *d*-methadone was examined using both current- and voltage-clamp recording. Intracellular recording of membrane potential indicated that both *l*- and *d*-methadone induced a maximal hyperpolarization suggesting that under these conditions both enantiomers of methadone were full agonists. The advantage of using intracel-

lular recording is that long-lasting, stable measurement of membrane potential is routine without the potential for rundown that often occurs with whole-cell recording. The maximum hyperpolarization is, however, limited by the approach of the membrane potential towards the potassium equilibrium potential. The maximum hyperpolarization will therefore overestimate agonist efficacy. Another consideration is the observation that the resting inward rectification (K_{IR}) was dramatically reduced by methadone, such that the amplitude of the hyperpolarization would be increased, in spite of a decrease in the opioid receptor-dependent current. Voltage-clamp recording using whole-cell recordings indicated that the maximum current induced by methadone was substantially less than that induced by ME. The current induced by *d*-methadone was less than *l*-methadone and both were less than that induced by ME. Different assays have concluded that methadone is a full (Selley *et al.*, 1998; Wallisch *et al.*, 2007; Rodriguez-Martin *et al.*, 2008) or a partial (Saidak *et al.*, 2006; Virk and Williams, 2008) agonist at μ-opioid receptors. The maximal efficacy of methadone ranged from 85% to 100% of the activation by DAMGO. The present results demonstrate that receptor reserve is assay dependent. That is, the maximum hyperpolarization was attained with methadone even though the outward current was less than the peak outward current induced by ME.

In the continued presence of a saturating concentration of methadone, the hyperpolarization peaked and declined to a greater extent than that observed with a saturating concentration of morphine or ME (Arttamangkul *et al.*, 2008). The decline in the amplitude of the hyperpolarization induced by prolonged exposure to a high concentration of methadone may result from either induction of desensitization or block of the potassium conductance. The mechanism(s) that account for acute opioid receptor-dependent desensitization have not been definitively identified. A component of the methadone-induced decline may result from a block of the $K_{IR}3$ channel conductance. The block of the $K_{IR}3$ conductance has a slow onset so the peak activation of $K_{IR}3$ channels may precede the onset of channel block. It is unlikely, however, that channel block accounts entirely for the decrease in the peak current because the time course of block of the current induced by activation of adrenoceptors was slower than the onset of opioid receptor-dependent desensitization.

Methadone/alkaloid blockade of ion channels

Ligands such as morphine, fentanyl, codeine and naloxone, have been shown to block NMDA receptors at high concentrations (K_i > 100 μM; Yamakura *et al.*, 1999). The morphine-induced block of NMDA receptors has been reported to be stereospecific, where *l*-morphine has a lower K_i value (160 μM) than *d*-morphine (4.7 μM; Stringer *et al.*, 2000). Morphine has also been reported to block $K_{IR}3$ channel conductance when applied at high concentrations, but the stereoselectivity of this action has not been examined (Blanchet *et al.*, 2003). The present results indicate however, that the block of NMDA receptors by *l*- and *d*-methadone was similar, confirming the lack of a stereoselective action (Choi and Viseskul, 1988; Gorman *et al.*, 1997; Ebert *et al.*, 1998). The greater conformational flexibility of methadone compared with other opiates was proposed to result in the non-stereospecific channel block (Pert and Snyder, 1973a). Previous studies have found that methadone blocked voltage-gated potassium channels by acting at an intracellular site, similar to the blockade induced by tetraethylamonium binding in the inner vestibule of voltage-gated potassium channels (Horrigan and Gilly, 1996). The block of $K_{IR}3$ channels may result from a similar mechanism. The slow time course of the block of $K_{IR}3$ channels may result from the slow penetration of methadone into the cytoplasm.

Clinical relevance and significance

Racemic methadone is prescribed for the treatment of opiate addiction and analgesia. The half-life of methadone is long enough to maintain the basal level of methadone so that it can be used on an outpatient basis (Dole and Nyswander, 1965; Dole and Kreek, 1973). The danger of methadone is the rapid partitioning into the brain resulting in high levels. The initiation of methadone maintenance therefore begins with low doses even in morphine-tolerant individuals (Dole and Nyswander, 1965). Studies in patients who are on methadone maintenance have an average plasma concentration of 700–800 nM (Kreek, 2000). When the distribution of methadone between plasma and CSF were compared, CSF levels were only a small fraction (2%–73%) of the plasma levels (Rubenstein *et al.*, 1978). Chronic treatment of rats with methadone also required initiation of treatment with low doses of methadone over a 2-day period before increasing the dose using osmotic minipumps (Virk *et al.*, unpublished). Rats that received (*l*/*d*) methadone (60 $mg \cdot kg^{-1} \cdot day^{-1}$) for 7 days had a plasma concentration that was in the same range (*l*-methadone – 309 nM, and *d*-methadone – 452 nM, Virk *et al.*, unpublished) as measurement in humans (Kreek, 2000). Lethal plasma levels of methadone in patients on maintenance therapy averaged 3 μM (Perret *et al.*, 2000). The results of this study suggest that the concentrations of methadone measured in

human studies do not reach values where there is a large decrease in $K_{IR}3$ channel or NMDA receptor-dependent conductance. Levels that approach the channel blocking concentrations are only described in overdose cases (Perret *et al.*, 2000). Studies in rodent models have shown that methadone is capable of decreasing NMDA receptor-induced firing in the spinal cord and NMDA receptor-dependent measures of pain (Davis and Inturrisi, 1999; Sotgiu *et al.*, 2009). While there is no question that methadone is capable of these actions, the concentrations that are achieved in the experiments carried out *in vivo* are difficult to relate to the present work. Our conclusion is therefore that most of the clinical actions of methadone are mediated by opioid receptors.

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Conflicts of interest

None of the authors has a conflict of interest.

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