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Permalink

https://escholarship.org/uc/item/1sm240tt

Journal

European Journal of Pharmacology, 702(1-3)

ISSN

0014-2999

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Publication Date

2013-02-01

DOI

10.1016/j.ejphar.2013.01.022

Copyright Information

Peer reviewed

Neuropharmacology and analgesia

Stereo-selective inhibition of spinal morphine tolerance and hyperalgesia by an ultra-low dose of the alpha-2-adrenoceptor antagonist efaroxan

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ARTICLE INFO

ABSTRACT

Ultra-low doses of alpha-2 (α_2)-adrenoceptor antagonists augment spinal morphine antinociception and inhibit tolerance, but the role of receptor specificity in these actions is unknown. We used the stereo-isomers of the α_2 adrenoceptor antagonist, efaroxan to evaluate the effect of receptor specificity on the induction of spinal morphine tolerance and hyperalgesia. Tail flick and paw pressure tests were first used to evaluate high dose efaroxan (12.6 µg) and its stereo-isomers on clonidine analgesia in intrathecally catheterized rats. Ultra-low doses of individual isomers (1.3 ng) were then co-administered with morphine (15 µg) to determine their effects on acute antinociceptive tolerance and hyperalgesia induced by low dose spinal morphine (0.05 ng). Results demonstrate that high dose (+) efaroxan antagonized clonidine-induced antinociception, while (−) efaroxan had minimal effect. In addition, an ultra-low dose of (+) efaroxan (1.3 ng), substantially lower than required for receptor blockade, inhibited the development of acute morphine tolerance, while (−) efaroxan was less effective. Racemic (±) efaroxan effects were similar to those of (+) efaroxan. Furthermore, low dose morphine (0.05 ng) produced sustained hyperalgesia in the tail flick test and this was blocked by co-injection of (+) but not (−) efaroxan (1.3 ng). Given the isomer-specific efaroxan effects and their different receptor potencies, we suggest that inhibition of opioid tolerance by ultra-low dose efaroxan involves a specific interaction with spinal α_2-adrenoceptors in this model. Likewise, inhibitory effects of adrenoceptor antagonists on morphine tolerance may be due to blockade of opioid-induced hyperalgesia.

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1. Introduction

Repeated systemic or spinal opioid administration produces analgesic tolerance (Christie, 2008), a phenomenon linked to the induction of progressive and latent hyperalgesia with a consequent loss of drug potency (Chu et al., 2006). Indeed, the outcome of analgesic tolerance is a complex phenomenon resulting in the activation of various opponent processes (Harrison et al., 1998; Zeng et al., 2006) including the onset of mechanisms leading to opioid-induced hyperalgesia (Chu et al., 2008) and opioid receptor desensitization (Connor et al., 2004).

Ultra-low dose of the opioid antagonist, naltrexone paradoxically inhibits development of opioid tolerance (Shen and Crain,

1997) (ultra-low dose is defined as a dose several log units lower than that required to produce functional antagonism at the respective receptors), a finding many other studies have replicated (Powell et al., 2002; Terner et al., 2006; McNaull et al., 2007). For example, ultra-low doses of competitive opioid receptor antagonists such as naltrexone have been shown to paradoxically augment spinal morphine analgesia and inhibit or reverse the development of this opioid tolerance (Powell et al., 2002; Chindalore et al., 2005; Mattioli et al., 2010). Interestingly, the ultra-low dose phenomenon is not restricted to opioid antagonists but is also produced by ultra-low dose adrenergic antagonists. Hence, we have shown that ultra-low dose α_2 adrenoceptor antagonists both prevent and reverse established analgesic tolerance to morphine (Milne et al., 2008), a finding subsequently confirmed by Lilius et al. (2012). We have shown that ultra-low doses of structurally diverse α_2-adrenoceptor antagonists (atipamezole, yohimbine, mirtazipine, and idazoxan) also increase the acute antinociceptive effects of morphine, block the induction of acute as well as chronic tolerance, and effectively reverse established tolerance to spinal morphine in tests of

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thermal and mechanical nociception (Milne et al., 2008). The basis of these unusual crossover effects of the adrenergic antagonists remains unknown, but may involve action on G-protein coupled receptor heteromeric complexes and/or conformational cross-talk (Jordan et al., 2003; Vilardaga et al., 2008).

In the present study, we aimed to determine whether the ultra-low dose effects of an α_2 receptor antagonist to block acute morphine tolerance and opioid-induced hyperalgesia are receptor-mediated by using stereo-selective isomers. Efaroxan is a potent and selective α_2-adrenoceptor antagonist whose dextro isomer $(+)$ shows greater potency and activity than the levo isomer $(-)$ at this receptor. The compound is a 2-ethyl-substituted idazoxan analog, possessing a dihydrobenzofuranyl ring instead of a benzodiazoxan ring. In α_2-adrenoceptor binding experiments on the human frontal cortex, IC_{50} values for efaroxan at the α_2-adrenoceptor were reported to be 27 ± 1.9 nM for the $(+)$ and $11,000 \pm 580$ nM for the $(-)$ enantiomer (Flamez et al., 1997). In rats, bearing 6-hydroxydopamine-induced lesions of the dopaminergic nigrostriatal pathway, stereo-selective facilitating effects of the $(+)$ enantiomer of efaroxan were demonstrated on circling behavior (Chopin et al., 1999). Thus, using the racemic $(\pm)$ efaroxan and its stereoisomers we investigated whether (i) $(+)$ and $(-)$ efaroxan produce stereo-selective antagonistic effects on clonidine-induced antinociception in the acute thermal and mechanical nociception tests, (ii) doses of efaroxan stereo-isomers substantially below those producing α-adrenoceptor antagonism block the development of acute spinal morphine tolerance, and (iii) ultra-low doses of efaroxan exhibit a stereo-selective action on hyperalgesia induced by a low dose of morphine (Crain and Shen, 2000; McNaull et al., 2007).

2. Materials and methods

2.1. Subjects

All experiments were performed on male Sprague–Dawley rats (250–300 g) obtained from Charles River Laboratories, Montreal QC, Canada following approval by the Queen's University Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care. Animals were given ad libitum access to food and water, and were maintained under a 12 h light/dark cycle at room temperature (21–23 °C). Animals were acclimatized for 3–4 days before surgery.

2.2. Intrathecal catheterization

Intrathecal catheters were implanted under halothane anesthesia using the method described by Yaksh and Rudy (1976). Briefly, the animal was placed prone in a stereotaxic frame, a small incision was made in the atlanto-occipital membrane of the cisterna magna and a polyethylene catheter (PE10; 7.5 cm) inserted through the opening such that the tip reached the lumbar enlargement of the spinal cord. To avoid potential interaction with the test compounds, rats did not receive an analgesic pre-surgery, but did receive lactated Ringer's solution to prevent dehydration (5 ml, s.c.) and 0.04 ml/100 g Tribrissen 24% s.c. peri-operatively. Following surgery and recovery from the anesthetic, rats were returned to their cage with food and water available ad libitum (soft food was provided to any rat that did not appear to be eating well). Animals were monitored daily to inspect general appearance and any animal that showed signs of distress such as matted hair, spontaneous vocalization upon handling, or showing visible neurological deficits (forelimb or hind limb paralysis) was sacrificed immediately. Nylabone chew toys and a section of polyvinyl chloride (PVC) tubing was

provided for environmental enrichment. Minor skin lesions were treated with gentocin violet solution. All animals were allowed to recover for 4–5 days prior to experimentation. Investigational drugs were injected, in a single blind fashion, through the rostral exteriorized portion of the catheter in a 10 μL volume and flushed with 10 μL of normal saline.

2.3. Nociception assessment

Following conditioning to the testing environment, antinociception was assessed using thermal tail flick and mechanical paw pressure tests. The tail flick test (D'Amour and Smith, 1941) measured the response to a brief thermal stimulus applied 5 cm from the base of the tail with the use of an antinociception meter (Owen et al., 1981). Time for tail removal from the thermal stimulus was recorded with baseline latency set at 2–3 s and a cut-off time of 10 s to prevent tissue damage. The paw pressure test measures response to a brief mechanical nociceptive stimulus applied to the dorsal hind paw using an inverted air-filled syringe connected to a pressure gauge (Loomis et al., 1987). Pressure was gradually increased until withdrawal occurred (baseline 70–90 mmHg, cut-off 300 mmHg) (Milne et al., 2008). All animals were habituated to the testing apparatus for at least 3 days prior to experimentation (Milne et al., 2008). Tail flick testing preceded paw pressure testing in each animal and prior experience has shown no significant interaction between responses in these tests (Loomis et al., 1987). In hyperalgesia experiments, only the tail flick test was utilized, using a lower stimulus intensity yielding a baseline latency response of 9–10 s (cut off 24 s), as holding and restraining these animals for paw pressure testing leads to increased variability in withdrawal thresholds such that data are inconsistent in demonstrating a mechanical hyperalgesic effect.

All behavioral testing was performed without knowledge of the treatments, and testing occurred between 0800 and 1400 h during the light cycle. Drug, drug isomer, and vehicle treatments were administered in the same experiment.

2.4. Experiment 1: effects of racemic efaroxan and its stereo-isomers on clonidine antinociception

To establish the antagonist effects of efaroxan at α_2 receptors, efaroxan (12.6 μg or 1.3 μg) was administered concomitantly with clonidine (13.3 μg) via intrathecal (i.t.) injection through chronically implanted catheters. The effect of stereo-selective enantiomers $(+)$ efaroxan (12.6 μg) and $(-)$ efaroxan (12.6 μg) on clonidine-induced antinociception was also determined in both the tail flick and paw pressure tests.

2.5. Experiment 2: effect of ultra-low dose efaroxan and its stereo-isomers on acute spinal morphine tolerance

Acute tolerance to spinal morphine was induced by administration of three successive injections of intrathecal morphine (15 μg) delivered at 90 min intervals (McNaull et al., 2007). Thermal and mechanical response thresholds were recorded prior to and following drug injection using the tail flick and paw pressure tests respectively. Latencies to respond were evaluated at 30-min intervals since peak morphine effect in the tail flick and paw pressure tests occurs at this interval following drug injection (Powell et al., 2002; Milne et al., 2008).

To determine the effects of ultra-low dose efaroxan on morphine tolerance racemic $(\pm)$ efaroxan (1.3 ng or 0.13 ng) or $(+)$ efaroxan (1.3 ng) or $(-)$ efaroxan (1.3 ng) were injected concomitantly with the first, second and third dose of intrathecal morphine in the acute morphine tolerance paradigm described
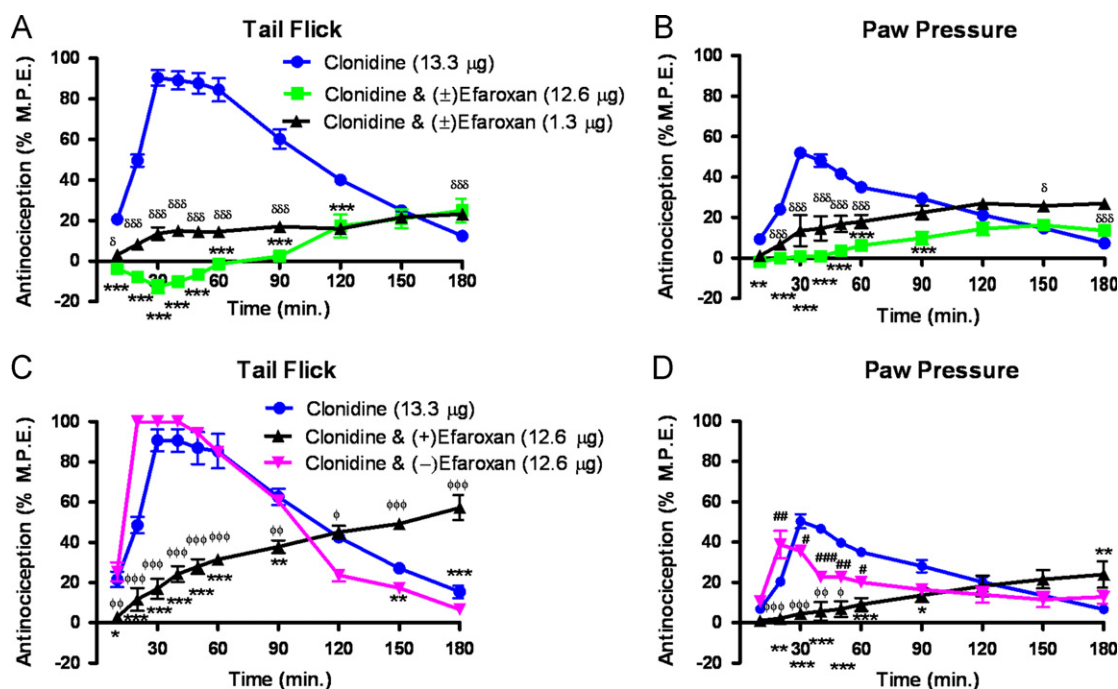
Fig. 1. Actions of racemic (±) efaroxan and its isomers on α_2 adrenoreceptor-mediated analgesia: Tail flick and paw pressure was assessed over a 180 min time course following concurrent intrathecal administration of 13.3 μg clonidine with the racemic mixture of efaroxan or its stereoisomers (1.3 μg or 12.6 μg) dissolved in sterile isotonic saline: (A) tail flick ($n=5$) and (B) paw pressure ($n=5$) tests. ***$=P < 0.001$, **$=P < 0.01$, for 12.6 μg (±) efaroxan and 13.3 μg clonidine compared to clonidine (13.3 μg) alone. $\delta\delta\delta=P < 0.001$, $\delta=P < 0.05$ for clonidine (13.3 μg) and 1.3 μg (±) efaroxan compared to clonidine (13.3 μg) alone. (C) Tail flick ($n=4$) and (D) paw pressure, ($n=4$) tests. ***$=P < 0.001$, **$=P < 0.01$, *$=P < 0.05$ for 12.6 μg (+) efaroxan and clonidine (13.3 μg) compared to clonidine (13.3 μg) alone. $\phi\phi\phi=P < 0.001$, $\phi\phi=P < 0.01$, $\phi=P < 0.05$ for (−) efaroxan (12.6 μg) and clonidine (13.3 μg) compared to (+) efaroxan (12.6 μg) and clonidine (13.3 μg). ###$=P < 0.001$, ##$=P < 0.01$, #$=P < 0.05$ for 12.6 μg (−) efaroxan and clonidine (13.3 μg) compared to clonidine (13.3 μg) alone.

above. The ability of efaroxan to attenuate acute morphine tolerance was determined by the ability of the drug to influence both the magnitude of the morphine-elicited response on day 1 of the testing period and on the morphine ED_{50} values obtained 24 h after repeated injections. Cumulative dose–response curves for the acute action of morphine were obtained 24 h after the repeated injections of morphine or morphine and efaroxan isomers to derive quantitative estimates of the opioid agonist potency (ED_{50} values). Dose–response curves were obtained by administering ascending cumulative doses of morphine (2.5, 5, 10 and 20 μg morphine in efaroxan (1.3 or 0.13 ng) or efaroxan plus morphine treatment groups and 12.5, 25, 50 and 100 μg morphine in the repeated morphine-treatment group) at 30 min intervals until a maximal antinociceptive response was obtained. Morphine ED_{50} values were derived from the dose–response curves obtained in these tests and calculated by linear regression using the Prism Graphpad software (version 4.0). The occurrence of antinociceptive tolerance was indicated by a progressive decrease in the magnitude of the antinociceptive effect produced by successive morphine injections (day 1), and a significant increase in the morphine ED_{50} value (day 2) reflecting a loss of the agonist potency (Milne et al., 2008).

2.6. Experiment 3: effects of ultra-low doses of efaroxan stereoisomers and other α_2-adrenoceptor antagonists on morphine hyperalgesia

Morphine hyperalgesia was induced by a single intrathecal injection of low dose morphine (0.05 ng) and analgesia assessed using the tail flick test (McNaull et al., 2007). In subsequent tests, morphine was co-injected with a dose (1.3 ng) of the efaroxan isomers evaluated in preceding experiments on tolerance, or with ultra-low doses of other α_2 receptor antagonists, atipamezole

(0.08 ng) or yohimbine (0.02 ng), previously found to modulate acute morphine tolerance (Milne et al., 2008).

2.7. Data analysis

All tail flick and paw pressure values were converted to percentage of maximum possible effect (M.P.E.) (M.P.E.$=100 \times$ ((post-drug response − baseline response)/(cutoff response − baseline response))). Data are expressed as mean $\pm$ S.E.M. for $N=4$–8 per group. ED_{50} values were determined using nonlinear regression analysis. A 2-way repeated-measures analysis of variance (ANOVA) with time as a within-subject factor and treatment as a between-subject factor was used to account for repeated measures design. Time X treatment interaction was included to test for differences in longitudinal response. Where applicable, Tukey's post-hoc tests were conducted.

2.8. Drugs

All drugs were dissolved in 0.9% sterile saline. Morphine sulfate (BDH Pharmaceuticals, Toronto, Canada), atipamezole (Farmos, Turku, Finland), yohimbine and clonidine (Sigma Chemical Co., St. Louis, MO, USA). The racemic efaroxan was obtained from Tocris Bioscience and efaroxan isomers were kindly provided by Dr. Marc Marien, Institut de Recherché Pierre Fabre, Castres, France.

3. Results

3.1. Actions of efaroxan and its stereoisomers on α_2-adrenoreceptor mediated analgesia

Intrathecal injection of clonidine (13.3 μg) produced an increase in withdrawal thresholds in both the tail flick and paw

pressure tests, with peak antinociceptive effects observed 30 min post-injection and a slow return towards baselines over the 180 min time course (Fig. 1A,B). The antinociceptive effects of clonidine were attenuated by co-administration of racemic ($\pm$) efaroxan (12.6 μg and 1.3 μg) compared to clonidine alone and this effect was greater with the higher dose (Fig. 1A, B). The effects produced by co-injection of stereo-isomers of efaroxan with clonidine demonstrate that ($+$) efaroxan (12.6 μg), the isomer with high binding affinity for α_2-adrenoceptors, inhibited clonidine-induced antinociception (Fig. 1C, D) in both the tail flick and paw pressure tests. In contrast, when the ($-$) isomer of efaroxan was co-injected with clonidine, the resulting antinociceptive effects in the tail flick test were not significantly different compared to clonidine alone (Fig. 1C). However, in the paw pressure test (Fig. 1D), inhibition occurred with the ($-$) isomer, but to a lesser extent than with the ($+$) isomer. There was shorter onset of analgesia with clonidine and the ($-$) efaroxan combination.

3.2. Action of racemic ($\pm$) efaroxan on acute morphine tolerance

Intrathecal administration of morphine (15 μg) produced an increase in withdrawal thresholds in both the tail flick and paw pressure tests. Three successive morphine injections administered at 90-min intervals represents a valid protocol for acute opioid antinociceptive tolerance (McNaull et al., 2007) (Fig. 2A).

Morphine co-administered with ultra-low dose racemic ($\pm$) efaroxan (1.3 ng) produced augmented antinociceptive effects at 60 min and 90 min following morphine injections at 90 and 180 min but there was no difference in peak antinociceptive effects at 30 min following each morphine injection compared to morphine alone (Fig. 2A). In the paw pressure test, morphine combined with ultra-low dose racemic ($\pm$) efaroxan produced sustained antinociceptive effects that were not different than peak antinociceptive effects of morphine at 30 min (Fig. 2B). Racemic ($\pm$) efaroxan alone (1.3 ng) did not produce significant effects on thermal tail flick latencies or paw withdrawal thresholds (Fig. 2A,B). Twenty-four hours following the acute morphine tolerance paradigm, all animals were exposed to cumulative injections of morphine to establish dose–response curves. Saline controls were not conducted for the current investigation but have been completed numerous times previously with no observed effect (Abul-Husn et al., 2007; Milne et al., 2008, 2011). Previous ED_{50} values obtained from cumulative dose–response curves in saline-treated rats were established at 5.5 and 5.9 μg for tail flick and paw pressure tests respectively (Milne et al., 2008). Co-treatment of animals with efaroxan (1.3 ng or 0.13 ng) significantly shifted the dose–response curves to the left indicating a reduction of antinociceptive tolerance in both the tail flick and paw pressure tests (Fig. 2C,D). The curve was apparently further left-shifted with 0.13 ng efaroxan
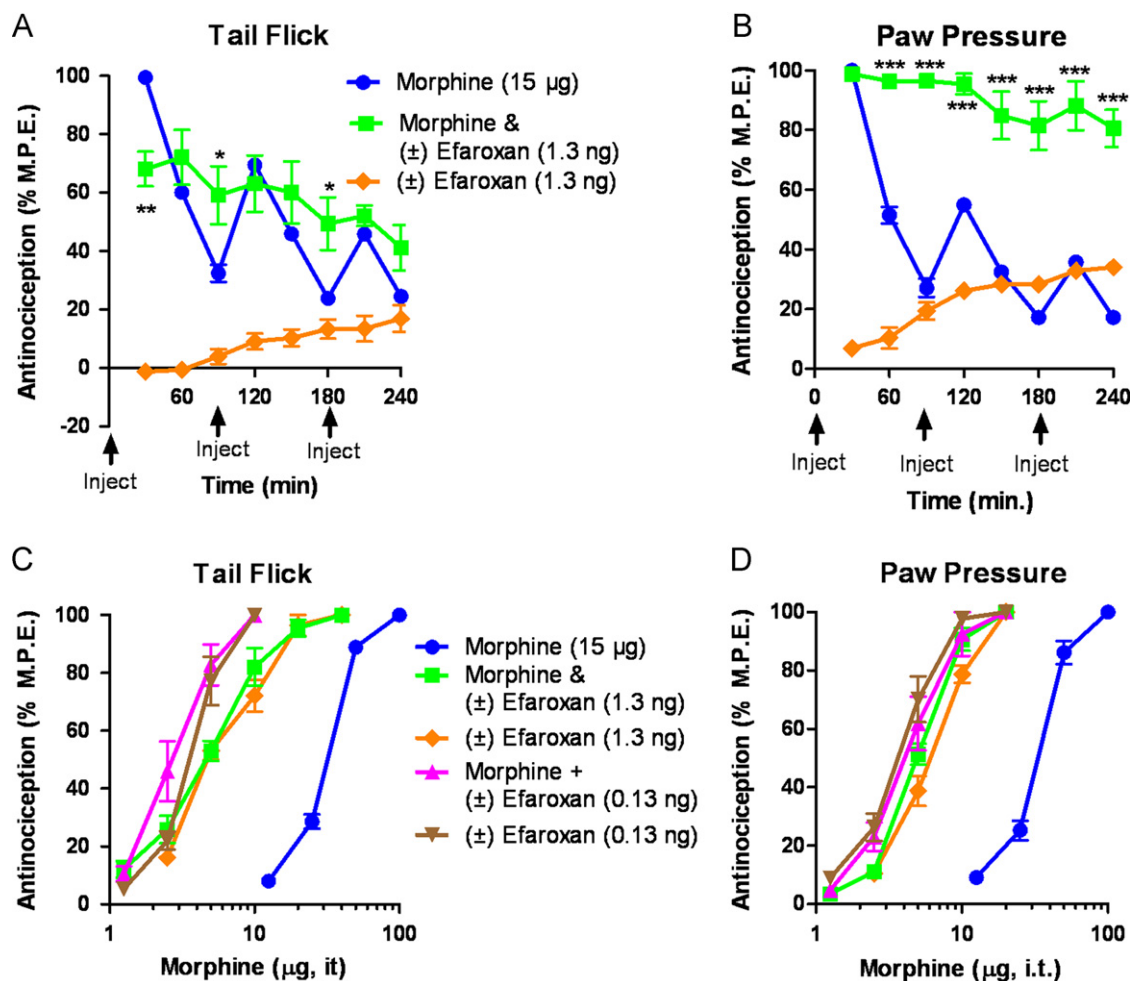
Fig. 2. Effect of racemic efaroxan on acute morphine tolerance: Tail flick (A) ($n=4$–8) and paw pressure (B) tests ($n=4$–8) were performed over a 240 min time course. Efaroxan injections were administered at 0, 90, and 180 min. Cumulative dose–response curves for tail flick (C) ($n=4$–8) and paw pressure (D) ($n=4$–8) tests were obtained by administering ascending cumulative doses of morphine at 30 min intervals until a maximal antinociceptive response was obtained. Morphine ED_{50} values were derived from the dose–response curves using linear regression analysis. ***$=P<0.001$, **$=P<0.01$, and *$=P<0.05$.

Table 1
Effect of low dose i.t. efaroxan and its stereoisomers on the induction of acute tolerance to morphine.

Treatment	Tail flick ED$_{50}$ (μg i.t.) (mean ± S.E.M.)	Paw pressure ED$_{50}$ (μg i.t.) (mean ± S.E.M.)
Fig. 2		
Morphine (15 μg)	28.92 ± 1.39	31.18 ± 1.62
Morphine (15 μg)+Efaroxan (1.3 ng)	4.42 ± 0.54[a]	4.79 ± 0.27[a]
Efaroxan (1.3 ng)	4.82 ± 0.47[a]	5.45 ± 0.31[a]
Morphine (15 μg)+Efaroxan (0.13 ng)	2.55 ± 0.36[a]	4.72 ± 1.30[a]
Efaroxan (0.13 ng)	3.60 ± 0.19[a]	3.60 ± 0.50[a]
Fig. 3		
Morphine (15 μg)	28.92 ± 1.39	31.18 ± 1.62
Morphine (15 μg)+(+) Efaroxan (1.3 ng)	2.46 ± 0.20[a,b]	3.5 ± 0.37[a,b]
Morphine (15 μg)+(−) Efaroxan (1.3 ng)	19.31 ± 0.42[a]	20 ± 1.06[a]
(+) Efaroxan (1.3 ng)	4.68 ± 0.43[a,b]	7.04 ± 0.74[a,b]
(−) Efaroxan (1.3 ng)	2.47 ± 0.28[a,b]	2.6 ± 0.30[a,b]

Morphine ED$_{50}$ values for the tail flick and paw pressure tests obtained from the cumulative dose–response curves in Figs. 2(C, D) and 3 (C, D) 24 h following termination of drug treatment. Data are presented as mean ± S.E.M.

[a] Significant difference from morphine (15 μg) group ($P < 0.001$).
[b] Significant difference from morphine (15 μg)+(−) efaroxan (1.3 ng) group ($P < 0.001$). $N = 4$–8 animals per treatment group.

compared to 1.3 ng efaroxan. Calculation of the ED$_{50}$ from the dose–response curves showed that the ED$_{50}$ of morphine (15 μg) alone was up to ten times higher than morphine co-administered with racemic (±) efaroxan (1.3 ng or 0.13 ng) or racemic (±) efaroxan alone in both the tail flick and paw pressure tests. Table 1 summarizes the ED$_{50}$ values from the dose–response curves in both the tail flick and paw pressure tests from Fig. 2.

3.3. Action of ultra-low dose efaroxan stereo-isomers on acute morphine tolerance

To determine if the effects of efaroxan were stereo-specific, both active and inactive isomers were co-injected with morphine in the acute morphine tolerance paradigm. In the thermal nociceptive test, co-administration of morphine (15 μg) with the (+) efaroxan isomer (1.3 ng) attenuated the loss of opioid anti-nociception throughout the 4-h time course, where after 4 h animals were still exhibiting significant antinociception (approximately 75–80% M.P.E., Fig. 3A). In contrast, co-administration with the (−) efaroxan isomer (1.3 ng) had no effect on the loss of opioid-induced antinociception in the acute tolerance paradigm in the thermal test (Fig. 3A). In the paw pressure test, co-administration of morphine with either the (+) efaroxan or the (−) efaroxan isomer significantly attenuated the loss of morphine antinociception throughout the time course. However, the effects of the (+) isomer were significantly different from those produced by the (−) isomer whereby the antinociceptive effects produced by co-injection of morphine with (+) efaroxan were significantly augmented compared to co-treatment with (−) efaroxan (Fig. 3B). Twenty-four h following the acute morphine tolerance paradigm all animals were exposed to cumulative injections of morphine to establish dose–response curves. Co-treatment of animals with (+) efaroxan significantly shifted the dose–response curves to the left indicating the prevention of antinociceptive tolerance (Fig. 3C,D). Co-treatment of animals with (−) efaroxan also significantly shifted morphine dose–response curves to the left although not as far left as non-opioid treated animals (Fig. 3C,D). Calculation of the ED$_{50}$ from the dose–

response curves showed that the ED$_{50}$ of morphine alone was significantly higher than morphine co-administered with (−) efaroxan isomer (1.3 ng) by approximately 50%, but was nearly ten-fold higher than morphine co-administered with (+) efaroxan isomer (1.3 ng) in both the tail flick and paw pressure tests. Table 1 lists the ED$_{50}$ values from the dose–response curves in both the tail flick and paw pressure tests from Fig. 3.

3.4. Attenuation of low dose morphine hyperalgesia with ultra low-dose alpha 2 receptor antagonists

To investigate changes in the development of opioid hyperalgesia, an ultra-low dose of morphine (0.05 ng) was administered intrathecally, alone or in combination with a low dose of an α_2 receptor antagonist. Opioid hyperalgesia was observed over the first 90 min after morphine injection (0.05 ng) as evidenced by a negative change in %M.P.E. After 120 min antinociceptive effects were produced with maximal effect observed 210 min post injection (Fig. 4A,B). Morphine co-administered with low dose (+) efaroxan stereo-isomer (1.3 ng) significantly attenuated morphine-induced hyperalgesia, while co-administration of the (−) efaroxan isomer (1.3 ng) had no effect (Fig. 4A). Interestingly, co-administration with an ultra-low dose of other α_2-adrenoceptor antagonists (atipamezole (0.08 ng) or yohimbine (0.02 ng)) also inhibited morphine-induced hyperalgesia causing analgesia at approximately 60 min after their administration (Fig. 4B). The maximal effect of morphine-induced antinociception was delayed but the maximal response achieved after 120 min was not influenced.

4. Discussion

The current investigation demonstrates that the α_2-adreno-ceptor antagonist efaroxan effectively inhibits the antinociceptive effects of clonidine in thermal and mechanical nociceptive tests. In addition, repeated acute administration of spinal morphine induced antinociceptive tolerance was effectively blocked by an ultra-low dose of the active (+) isomer of efaroxan. The dose that was effective in attenuating the development of antinociceptive tolerance also suppressed thermal hyperalgesia elicited by a low dose of intrathecal morphine.

The present study used a model of acute morphine tolerance and efaroxan stereo-isomers (with different receptor affinities) to investigate whether the actions of an α_2-adrenoceptor antagonist in modulating opioid analgesia and tolerance are due to interaction at the α_2-adrenoceptor. In the present study, ultra-low doses of the α_2 antagonist efaroxan inhibited the development of acute morphine tolerance, an effect reflected in both the maintenance of the opioid-induced response to repeated drug administration and prevention of the loss of agonist potency. Previous evidence implicating α_2 receptors in opioid analgesic tolerance was based on the actions of atipemazole, a highly selective α_2 antagonist (Milne et al., 2008, Lilius et al., 2012) and reinforced by replication of these major findings with other ligands including yohimbine, idazoxan and mirtazipine that are less selective, but have the common ability to block α_2 receptors (see Milne et al., 2008). The present study demonstrates that the effects of ultra-low dose efaroxan were stereo-selective in the thermal acute tolerance test, suggesting that the inhibition of tolerance by ultra-low doses of the antagonist indeed involves a specific interaction with spinal α_2-adrenoceptors. In this study, efaroxan was chosen because of strong evidence for its stereo-selectivity in both binding studies (Flamez et al., 1997) and in in vivo studies not involving nociception (Chopin et al., 1999).
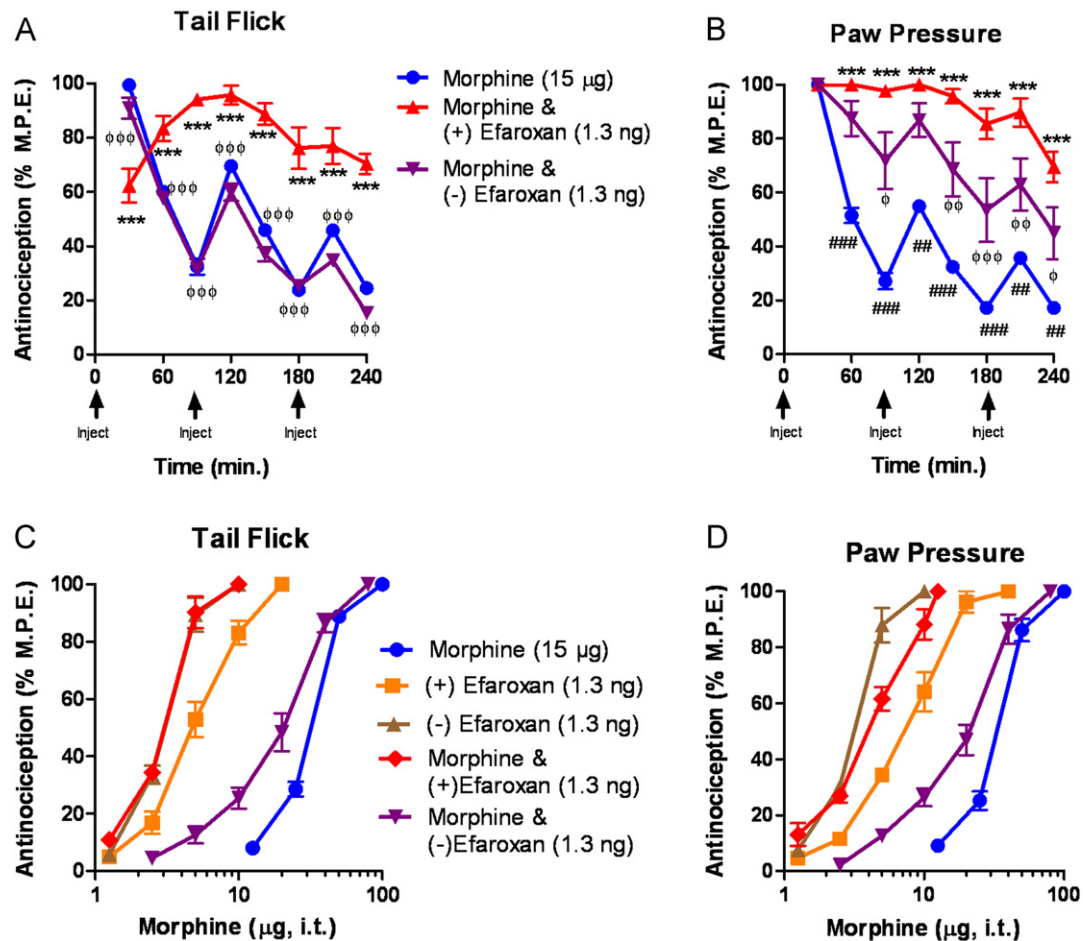
Fig. 3. Effect of ultra-low dose efaroxan stereoisomers on acute morphine tolerance: Tail flick (A) (n=7–8) and paw pressure (B) (n=7–8) tests were performed over a 240 min time course with efaroxan injections administered at 0, 90, and 180 min. The tail flick (C) (n=4–8) and paw pressure (D) (n=4–8) cumulative dose–response curves for the acute action of morphine were obtained 24 h later by administering ascending cumulative doses of morphine at 30 min intervals until a maximal antinociceptive response was obtained. Morphine ED_{50} values (n=4–8) were derived from the dose–response curves using linear regression analysis. ***=$P < 0.001$, **=$P < 0.01$, *=$P < 0.05$ for (+) efaroxan (1.3 ng) and morphine (15 µg) compared to morphine (15 µg) alone. $\phi\phi\phi$=$P < 0.001$, $\phi\phi$=$P < 0.01$, ϕ=$P < 0.05$ for (−) efaroxan (1.3 ng) and morphine (15 µg) compared to (+) efaroxan (1.3 ng) and morphine (15 µg), ###=$P < 0.001$, ##=$P < 0.01$, #=$P < 0.05$ for (−) efaroxan (1.3 ng) and morphine (15 µg) compared to morphine (15 µg) alone.

Overall, the data demonstrate that the development of both tolerance and hyperalgesia is largely stereo-specific, suggesting the effects of efaroxan were produced by inhibition of α_2-adrenoceptors. While the stereo-selectivity displayed by pharmacological systems constitutes the best evidence that receptors exist and that they incorporate concrete molecular entities as integral components of their active sites (Lehmann, 1982), the stereo selective action of isomers is not absolute. This may explain some of the partial effects of (−) efaroxan in its effect on acute morphine tolerance in the paw pressure test. (−) efaroxan is the "less active" isomer as reflected in binding studies (Flamez et al., 1997). The use of ultra-low doses, however, potentially calls into question whether the effects are mediated *via* activity at adrenoceptors, since much larger doses are required to antagonize an adrenergic agonist. While stereo-selectivity is confirmed, the precise receptor remains elusive in the absence of binding studies to confirm significant binding affinity with α_2-adrenoceptors at such low concentrations, as those used in our experiments.

Ultra-low dose racemic efaroxan alone produced delayed analgesia in the paw pressure test at 240 min. We do not have a good explanation for this effect although the delayed antinociception may possibly be due to an interaction of efaroxan with endogenous opiates released during repeated testing. Interestingly, BRL 44408, a highly selective α_{2A} adrenoceptor antagonist has been recently

shown to exhibit analgesia in a model of visceral pain. The authors (Dwyer et al., 2010) suggest that selective α_{2A}-adrenoceptor antagonism, (either by direct inhibition of α_{2A} autoreceptors or through a heteroceptor function of α_{2A}-adrenoceptors) may be useful in pain therapy. It is conceivable that efaroxan may have similar actions.

The mechanism by which ultra-low dose α_2 antagonists inhibit the development of acute morphine tolerance is unknown, although it is well accepted that there are interactions between these G-protein coupled receptors. Thus, agonists of mu opioid and α_2 receptors produce a synergistic effect in that the activation with α_2 agonists augments opioid-induced antinociception in rodents (Fairbanks et al., 2002; Tajerian et al., in press), and such combination has been proven beneficial in clinical practice whereby effective pain treatment was reported with reduced side effects when clonidine was combined with an opioid agonist (Eisanach et al., 1994; Paech et al., 2004). Additionally, morphine-induced antinociception recruits α_2 receptors as demonstrated by reduced analgesic potency in α_{2A} null mutant mice (D79N point mutation) compared to wild type animals (Stone et al., 1997). In addition to functional synergistic interactions, mu opioid receptors have been shown to form heteromers with several G protein coupled receptors involved in pain regulation including the α_2 receptors (Gupta et al., 2006; Jordan et al., 2003). Such interactions have been reported to occur not only in the spinal cord but
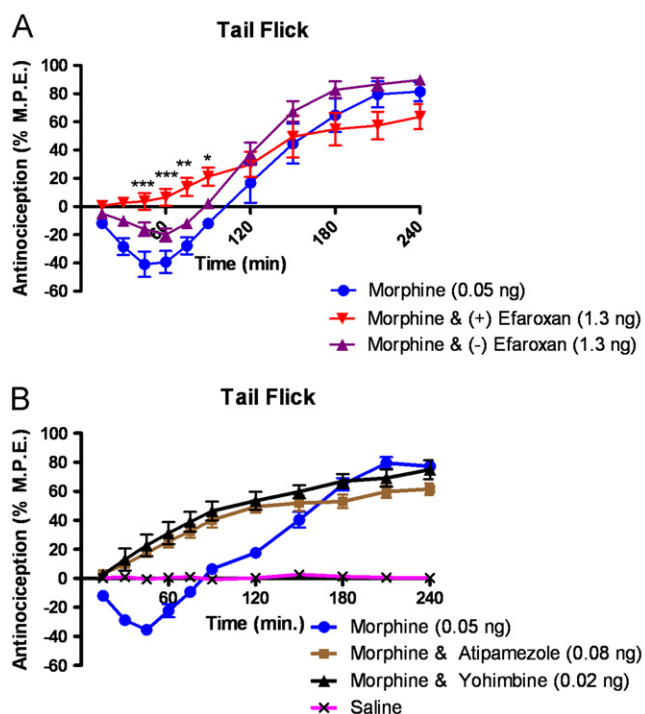
Fig. 4. Attenuation of morphine hyperalgesia with low dose α_2 antagonists: Thermal tail flick responses were assessed over a 240 min time course following a single intrathecal injection of low dose morphine (0.05 ng) either alone or co-administered with an ultra-low dose of efaroxan isomers (1.3 ng) (A) ($n=5$–6) or other α_2 receptor antagonists, atipamezole (0.08 ng) or yohimbine (0.02 ng) (B) ($n=5$–6). ***=$P<0.001$, **=$P<0.01$, *=$P<0.05$ compared to morphine (0.05 ng).

also at the level of the primary afferent neurons and other CNS sites (Illes and Norenberg, 1990). These receptors, either singly or as a heterodimer, activate common signal transduction pathways mediated through the inhibitory G proteins (G (i) and G (o)). However, there is evidence that continued opioid exposure of neurons in culture (Crain and Shen, 1996) or prolonged administration of opioids *in vivo* (Crain and Shen, 2000) could paradoxically produce facilitatory effects *via* activation of stimulatory G proteins (G(s)). Similarly, the hyperalgesic effects produced by low dose intrathecal morphine may also involve opioid receptor activation of Gs (McNaull et al., 2007; Esmaeili-Mahani et al., 2008). One of the mechanisms that may underlie the effects of the ultra-low dose α_2 antagonists is to prevent the mu opioid receptor from coupling to stimulatory effector systems that are initiated *via* activation of Gs. Such an effect could account for the ability of the stereo-selective effects of efaroxan to block the induction of thermal hyperalgesia resulting from a low dose of spinal morphine, as well as acute opioid tolerance. Alternatively, conformational cross-talk controlling cell signaling between α_2 and mu-opioid receptors (Vilardaga et al., 2008) may allow for the ultra-low dose of α_2 antagonists to augment the interaction of the mu receptor with its ligand. It is also relevant to consider the possibility of efaroxan producing its effects *via* the imidazoline receptor. Hence, clonidine is an agonist at α_2 as well as imidazoline receptors (Reis and Piletz, 1997), however both stereo-isomers of the alkoxy-substituted imidazoline derivative efaroxan displays low affinity for imidazoline receptors (Vauquelin et al., 1999), thus making it unlikely that this would be a potential mechanism for the effects produced in the present study.

It is also worth considering the aspect of opioid-induced hyperalgesia and whether such phenomenon occurs in an acute opioid tolerance model. Previous studies have suggested the induction of hyperalgesia as a contributing factor in the development of acute opioid tolerance (see McNaull et al., 2007). Many mechanisms have been proposed to mediate opioid-induced hyperalgesia (Lee et al., 2011; Angst and Clark, 2006) and involve the activation of opponent processes (Bryant et al., 2005). A single dose of morphine (Goldfarb et al., 1978) or heroin (Celerier et al., 2001) can generate naloxone-precipitated hyperalgesia that has been replicated in non-addicted humans following a single injection of morphine (Compton et al., 2003). Similar effects are reported following remifentanil infusion for anesthesia (Guignard et al., 2000). Under these conditions, hyperalgesia has been associated with increased amplitude of spinal cord reflexes (Goldfarb et al., 1978) and increased activity of nociceptive facilitatory neurons in the medulla (Neubert et al., 2004), each of which effectively results in increased pain behaviors. Hence, the doses of morphine used in the acute tolerance study may recruit opponent processes that initiate a hyperalgesic state, and whether such mechanisms are similar to low-dose morphine-induced hyperalgesia remains unknown. However, ultra-low dose α_2 antagonists appear to mitigate the genesis of such processes.

It is noteworthy that the less active stereoisomer of efaroxan partially inhibited the development of opioid tolerance in the mechanical nociceptive test and partially shifted the dose response curve for morphine following the acute tolerance paradigm. This is consistent with stereo-selectivity not being absolute as stated previously and may potentially explain the absence of complete stereo-specificity across the tests. There is no evidence to suggest that mechanisms of opioid tolerance differ between nociceptive modalities, however, it is not uncommon that opioid-induced mechanical hyperalgesia is reported more often than warm thermal hyperalgesia in clinical studies of healthy human subjects (Schmidt et al., 2007). Thus, mechanical tests may be more sensitive to detect the presence of opioid-induced hyperalgesia. In the present study, all animals were catheterized for spinal delivery of drugs and such catheterization causes neuro-inflammation on its own (DeLeo et al., 1997) and can facilitate the development of opioid tolerance (Mattioli et al., 2012). Therefore, the catheter-induced neuro-inflammation may have sensitized nociceptive neurons that facilitated opioid-induced hyperalgesia in the acute opioid tolerance model.

5. Conclusion

The present study shows concomitant administration of an exceedingly low dose of an α_2 antagonist can inhibit the development of acute opioid analgesic tolerance and low dose morphine-induced thermal hyperalgesia in a stereo-selective manner. This result suggests that the effects are indeed *via* an interaction between the opioid and adrenergic system rather than an alternative receptor pathway. It is not known if such processes occur in a chronic tolerance model although our previous experiments have demonstrated the ability of similar low doses of diverse, chemically distinct α_2 antagonists to prevent and reverse established antinociceptive tolerance following chronic morphine administration (Milne et al., 2008). It is also unknown if this interaction is specific to spinal sites and merits further investigation as to whether systemic administration of these ligands will produce similar effects.

Role of funding source

Acknowledgments

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