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The α_{2A}-adrenoceptor subtype is not involved in inflammatory hyperalgesia or morphine-induced antinociception

Janne Lähdesmäki[a], Mika Scheinin[a], Antti Pertovaara[b], Heikki Mansikka[c,*]

[a] Department of Pharmacology and Clinical Pharmacology, University of Turku, Turku, Finland
[b] Department of Physiology, University of Turku, Turku, Finland
[c] Department of Anesthesiology and Intensive Care, Turku University Hospital, P.O. Box 52, FIN-20521, Turku, Finland

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Abstract

The purpose of the present study was to investigate the role of the α_{2A}-adrenoceptor subtype in inflammatory hyperalgesia, and in adrenergic–μ-opioid interactions in acute pain and inflammatory hyperalgesia. Behavioral responses to mechanical and thermal stimuli were studied in α_{2A}-adrenoceptor knockout mice and their wild-type controls. Thermal nociception was evaluated as paw withdrawal latencies to radiant heat applied to the hindpaws. Mechanical nociception was measured using von Frey monofilament applications to the hindpaws. Mechanical and thermal hyperalgesia, induced with intraplantar carrageenan (1 mg/40 μl) were compared in α_{2A}-adrenoceptor knockout and wild-type mice. The effects of the systemically administered μ-opioid receptor agonist morphine (1–10 mg/kg) were evaluated on mechanical withdrawal responses under normal and inflammatory conditions in knockout and wild-type mice. Withdrawal responses to radiant heat and von Frey monofilaments were similar in α_{2A}-adrenoceptor knockout and wild-type mice before and after the carrageenan-induced hindpaw inflammation. Also, the antinociceptive effects of morphine in mechanical nociceptive tests were similar before and after carrageenan-induced hindpaw inflammation. Our observations indicate that α_{2A}-adrenoceptors are not tonically involved in the modulation of inflammation-induced mechanical and thermal hyperalgesia. In addition, α_{2A}-adrenoceptors do not appear to play an important role in μ-opioid receptor-mediated antinociception or antihyperalgesia.

Keywords: Pain; Hyperalgesia; Carrageenan; α_2-Adrenoceptor subtype; Morphine

1. Introduction

Previous studies indicate that α_2-adrenoceptor and μ-opioid receptor have an important role in pain modulation during physiological and inflammatory conditions (Hylden et al., 1991; Idänpään-Heikkilä et al., 1994; Kayser and Guilbaud, 1983; Mansikka and Pertovaara, 1995; Stanfa et al., 1992). Both α_2-adrenoceptor agonists and μ-opioid receptor agonists have increased antinociceptive potency in inflammatory conditions. Several studies indicate synergistic interactions between α_2-adrenoceptor agonist- and μ-opioid receptor agonist-mediated antinociceptive effects (Ossipov et al., 1989; Sullivan et al., 1987; Wilcox et al., 1987). α_2-Adrenoceptor antagonists have been shown to reverse the antinociceptive effects of morphine on acute and inflammatory pain models, indicating involvement of α_2-adrenoceptors in μ-opioid receptor agonist-induced antinociception (Browning et al., 1982; Herrero and Solano, 1999; Hylden et al., 1991; Ossipov et al., 1989). However, Stanfa and Dickenson (1994) were not able to block the enhanced antinociceptive potency of opioids in suppressing C-fiber evoked responses of spinal dorsal horn neurons by α_2-adrenoceptor antagonists during inflammation. In a recent study (Mansikka et al., 2002), we showed that μ-opioid receptor activation is not required for α_2-adrenoceptor-induced antinociception in acute pain, but μ-opioid receptors have an important role in the antihyperalgesic actions of α_2-adrenoceptor agonists in inflammatory pain.

The three α_2-adrenoceptor subtypes (α_{2A}, α_{2B} and α_{2C}) have different and distinct tissue distribution patterns both in the central nervous system and in the periphery (McCune et al., 1993; Nicholas et al., 1996; Scheinin et al., 1994;

* Corresponding author. Tel.: +358-2-3138135129; fax: +358-2-2502610.
E-mail address: heikki.mansikka@tyks.fi (H. Mansikka).

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Wang et al., 1996). Previous studies have indicated that α_{2A}-adrenoceptors mediate the antinociceptive effect of α_2-adrenoceptor agonists and the spinal adrenergic–opioid synergy (Hunter et al., 1997; Lakhlani et al., 1997; Stone et al., 1997). The role of the α_{2A}-adrenoceptor subtype in neuropathic pain has been studied by Malmberg et al. (2001) and Kingery et al. (2000). In the study by Kingery et al., the authors concluded that α_{2A}-adrenoceptors contribute to the development of neuropathic heat hyperalgesia, whereas the study by Malmberg et al. indicated that α_{2A}-adrenoceptors are not required for the development of neuropathic pain. Li and Eisenach (2001) showed that α_{2A}-adrenoceptor activation reduces capsaicin-induced glutamate release, indicating that α_{2A}-adrenoceptor might be involved in the modulation of neurogenic pain. There is, however, no experimental evidence on the role of the α_{2A}-adrenoceptor subtype in the modulation of inflammatory pain. Also, there is a limited amount of data on the interaction of α_{2A}-adrenoceptors and μ-opioid receptors in the modulation of pain and inflammatory hyperalgesia. Since there are no subtype-selective agonists for α_{2A}-adrenoceptor, we used mice with a targeted inactivation of the α_{2A}-adrenoceptor gene. The purpose of the present study was to investigate the role of α_{2A}-adrenoceptors in inflammatory pain by comparing the development of carrageenan-induced hyperalgesia in α_{2A}-adrenoceptor knockout versus wild-type mice. Also, the interaction between α_{2A}-adrenoceptors and μ-opioid receptors was assessed by determining the effect of the μ-opioid receptor agonist morphine on acute pain and inflammatory hyperalgesia in α_{2A}-adrenoceptor knockout and wild-type mice.

2. Materials and methods

The experiments were performed using α_{2A}-adrenoceptor knockout and wild-type control mice. A total of 30 wild-type and 30 knockout (15 male and 15 female animals in each genotype group) mice of 15–21 weeks of age were used. Mice deficient in α_{2A}-adrenoceptors were generated by gene targeting, which has been described previously (Altman et al., 1999). Age-matched wild-type C57BL/6J mice of the same genetic background (Jackson Laboratories, Bar Harbor, ME) were used as control animals. The animals were maintained on a 12-h light/dark cycle and were provided with food and water ad libitum. The α_{2A}-adrenoceptor knockout mice have multiple subtle behavioral alterations in detailed scrutiny, although the mice appear grossly normal and have no abnormalities, for example, in general development or breeding capability (Lähdesmäki et al., 2002). Knockout animals could not be distinguished from the control mice by their appearance. For all behavioral experiments, the animals were allowed to habituate for 5 days to the testing environment and for 15 min each day before the actual testing was started. The research protocol was approved by the Institutional Animal Care Committee of the University of Turku and by the Regional Government of Western Finland. All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data.

2.1. Withdrawal frequencies to mechanical stimuli

Mechanical withdrawal frequencies were measured using calibrated von Frey monofilaments (Stoelting, Wood Dale, IL, USA). Mice were placed in a plexiglass chamber on an elevated mesh screen. Six von Frey monofilaments, producing forces of 0.06, 0.20, 0.52, 1.15, 2.86 and 4.43 g, were applied to the plantar surface of each hind paw in an ascending series. Each monofilament was applied five times for approximately 1 s. The frequency of paw withdrawal (%) was calculated for each monofilament.

2.2. Behavioral responses to thermal stimuli

Radiant heat (Plantar test apparatus, Ugo Basile, Varese, Italy) was applied from below to the plantar surface of each hind paw and the withdrawal latency was measured with an electronic timer (Hargreaves et al., 1988). Three measurements were performed on each hind paw with at least 1-min intervals to determine mean withdrawal latency.

2.3. Behavioral responses to morphine and naloxone

The effect of morphine, a μ-opioid receptor agonist, on mechanical withdrawal frequencies was tested in homozygous α_{2A}-adrenoceptor knockout and wild-type mice. A cumulative drug dosing regimen was used (1, 3 and 10 mg/kg of morphine, s.c.). After the last morphine dose, animals received a s.c. injection of naloxone, a μ-opioid receptor antagonist (1 mg/kg). Responses to mechanical stimuli were tested 20 min following each drug injection. The interval between drug injections was 40 min.

2.4. Experimental protocol

There were three experimental groups in this study. All experimental groups consisted of α_{2A}-adrenoceptor knockout and wild-type control mice with an equal number of animals of each genotype ($n = 10$, consisting of 5 males and 5 females). Each animal was studied in only one experiment and the mice were killed immediately after the experiments.

(1) Drugs only group: the effects of morphine and naloxone were studied on mechanical responses.
(2) Carrageenan only group: mechanical and heat responsivity were determined before and after intraplantar injection of carrageenan (1 mg/40 μl) to the hind paw. Mechanical and heat responsivity was assessed 2, 4, 6 and 24 h following ipsilateral carrageenan injection.

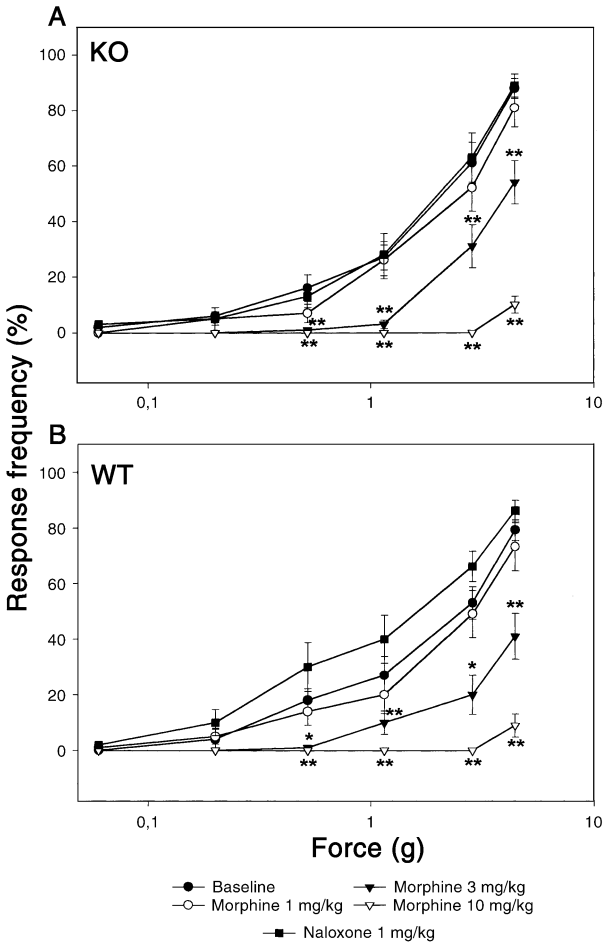

Fig. 1. Paw withdrawal frequencies to von Frey monofilaments following s.c. cumulative dosing of morphine and naloxone in intact mice. Morphine dose-dependently decreased paw withdrawal responsivity. This effect was reversed by naloxone. There were no significant differences in withdrawal responses between (A) α_{2A}-AR knockout (KO) and (B) wild-type (WT) mice before or after the cumulative drug dosing (Mann–Whitney's *U*-test, $p>0.05$, $n=10$ in KO group, $n=10$ in WT group; $*p<0.05$, $**p<0.01$ compared to predrug values, Wilcoxon's matched pairs test).

(3) Carrageenan + drugs group: the effect of cumulative dosing of morphine followed by naloxone was studied on mechanical responsivity starting at 2 h after intraplantar carrageenan injection.

2.5. Statistical analysis

Withdrawal responses evoked by mechanical stimuli were analyzed with Friedman's analysis of variance (ANOVA) followed by Wilcoxon's matched pairs and Mann–Whitney's *U*-tests. Radiant heat response latencies were analyzed with repeated measures two-way ANOVA followed by Tukey's test. The software package Statistica (StatSoft, Tulsa, OK, USA) was used in all statistical analyses. $P<0.05$ was considered to be significant. Data are presented as means ± S.E.M. unless otherwise indicated.

3. Results

3.1. The effect of morphine and naloxone on mechanical withdrawal responsivity (Protocol 1)

Cumulative dosing of morphine dose-dependently attenuated the paw withdrawal responses to mechanical stimuli. This effect was reversed by naloxone (1 mg/kg, s.c.). There were no significant differences in mechanical withdrawal responses between α_{2A}-adrenoceptor knockout and wild-type mice before or after the cumulative drug dosing (Fig. 1).

3.2. The effect of carrageenan-induced inflammation on mechanical and thermal withdrawal responsivity (Protocol 2)

Mechanical and heat responses were studied 2, 4, 6 and 24 h after intraplantar carrageenan (1 mg/40 μl)

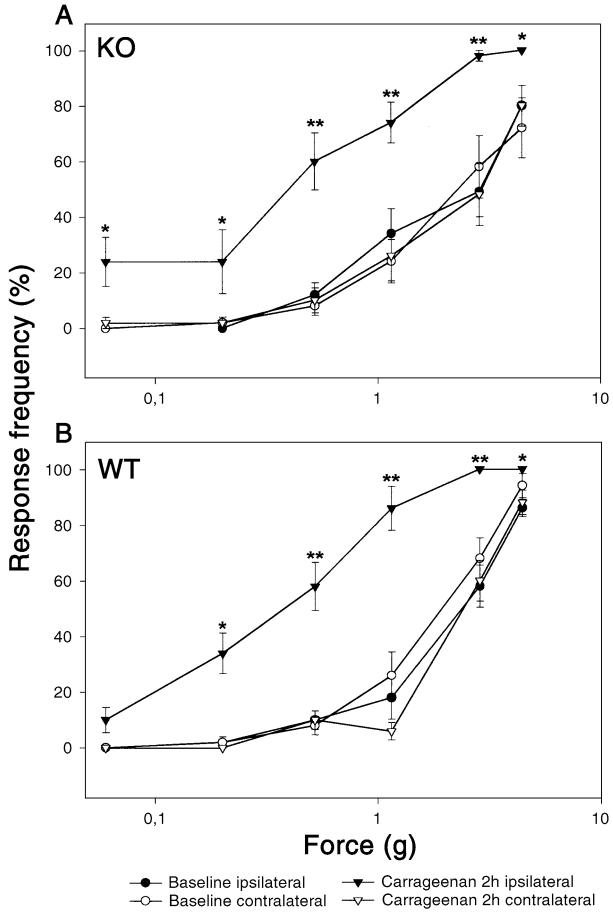

Fig. 2. Paw withdrawal frequencies to von Frey monofilaments following intraplantar carrageenan (1 mg/40 μl). Carrageenan-induced inflammatory hyperalgesia was assessed 2, 4, 6 and 24 h after injection. There were no differences in carrageenan-induced mechanical hyperalgesia at any of time point between (A) α_{2A}-AR knockout (KO) and (B) wild-type (WT) mice (Mann–Whitney's *U*-test, $p>0.05$, $n=10$ in KO group, $n=10$ in WT group; $*p<0.05$, $**p<0.01$ compared to precarrageenan values, Wilcoxon's matched pairs test). For clarity, data are only presented for the 2-h time point.

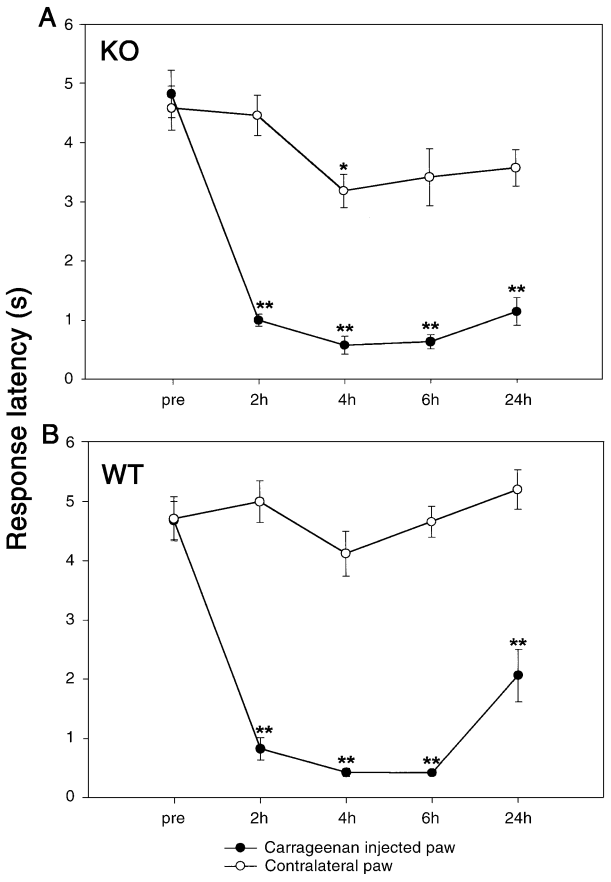

Fig. 3. Paw withdrawal latencies to radiant heat following intraplantar carrageenan (1 mg/40 μl). Paw withdrawal latencies decreased similarly following intraplantar carrageenan in (A) α_{2A}-AR knockout (KO) and (B) wild-type (WT) mice (ANOVA, $p>0.05$, $n=10$ in KO group, $n=10$ in WT group, $^{*}p<0.05$, $^{**}p<0.01$ compared to precarrageenan values, Tukey's test).

injections. Carrageenan-induced paw inflammation enhanced mechanical withdrawal responsivity significantly already at 2 h after carrageenan injection similarly in α_{2A}-adrenoceptor knockout and wild-type mice (Fig. 2). Paw inflammation also decreased the withdrawal latencies to radiant heat in both α_{2A}-adrenoceptor knockout and wild-type mice (Fig. 3). The enhanced responses to mechanical and thermal stimuli remained unchanged until the end of the 24-h observation period. For the sake of clarity, the mechanical withdrawal responses are presented only for the 2-h time point. There were no differences in carrageenan-induced mechanical or thermal hyperalgesia between α_{2A}-adrenoceptor knockout and wild-type mice at any of the investigated time points. Response latencies in the contralateral paw decreased in α_{2A}-adrenoceptor knockout mice at the 4-h time point following carrageenan compared to baseline, but there were no differences between the genotypes (Fig. 3).

3.3. The effect of cumulative dosing of morphine on carrageenan-induced mechanical hyperalgesia (Protocol 3)

The effect of cumulative dosing of morphine (1–10 mg/kg, s.c.) was studied on mechanical responsivity starting at 2 h after intraplantar carrageenan injection. Carrageenan-induced mechanical hyperalgesia was similar in α_{2A}-adrenoceptor knockout and wild-type mice before morphine administration. Cumulative dosing of morphine dose-dependently attenuated the paw withdrawal responsivity to mechanical stimuli. There were no significant differences in mechanical withdrawal responses between α_{2A}-adrenoceptor knockout and wild-type mice before or after the cumulative drug dosing (Fig. 4). The effects of morphine on the mechanical responses of the contralateral paw were also

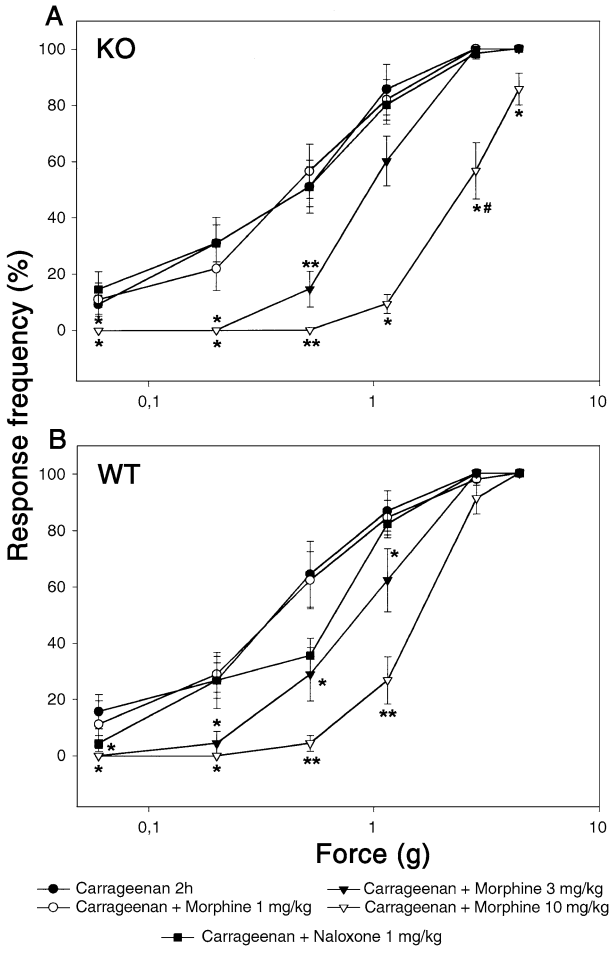

Fig. 4. Paw withdrawal frequencies to von Frey monofilaments following cumulative dosing of s.c. morphine and naloxone after intraplantar carrageenan (1 mg/40 μl). Data are presented from the side of the inflammation. There was no difference in the paw withdrawal frequencies 2 h after intraplantar carrageenan injection between (A) α_{2A}-AR knockout (KO) and (B) wild-type (WT) animals (Mann–Whitney's U-test, $p>0.05$, $n=10$ in KO group, $n=10$ in WT group). Morphine dose-dependently attenuated carrageenan-induced mechanical hyperalgesia similarly in α_{2A}-AR knockout and wild-type mice ($^{\#}p<0.05$ KO vs. WT, Mann–Whitney's U-test; $^{*}p<0.05$, $^{**}p<0.01$, compared to values obtained 2 h following carrageenan injection, Wilcoxon's matched pairs test).

similar between the genotypes (data not shown). In addition, naloxone reversed the effects of morphine in the knockout and wild-type mice in both intact (Fig. 1) and inflammatory (Fig. 4) conditions in a similar fashion.

4. Discussion

The main results from the present study are that (1) the lack of α_{2A}-adrenoceptors does not influence the development or maintenance of mechanical or thermal hyperalgesia during inflammation and (2) α_{2A}-adrenoceptors do not influence μ-opioid receptor-mediated antinociception in physiological or inflammatory conditions.

The deletion of the gene encoding the α_{2A}-adrenoceptor was verified with PCR. We have also shown that the binding of the α_2-adrenoceptor specific but subtype non-selective antagonist [^{3}H]RS-79948-197 is almost totally abolished (e.g. 83% reduction in B_{max} in cortex) in the brains of α_{2A}-adrenoceptor knockout mice. The remaining [^{3}H]RS-79948-197 binding is limited to brain regions known to contain the α_{2C}-adrenoceptor, such as the caudate–putamen nucleus (Lähdesmäki et al., 2002). Consequently, the behavioural (inhibition of locomotor activity), physiological (reduction of body temperature) and neurochemical (inhibition of noradrenaline metabolism) responses to the α_2-adrenoceptor agonist dexmedetomidine are almost totally absent or in α_{2A}-adrenoceptor knockout mice (Lähdesmäki et al., in press).

4.1. The role of α_{2A}-adrenoceptors in inflammatory hyperalgesia

Previous studies using non-subtype selective α_2-adrenoceptor agonists have indicated that α_2-adrenoceptors play an important role in pain modulation during acute pain and inflammatory hyperalgesia (Hylden et al., 1991; Idänpään-Heikkilä et al., 1994; Mansikka and Pertovaara, 1995). These studies have also demonstrated that α_2-adrenoceptor agonists have increased antinociceptive potency during inflammatory pain. Several studies have, indeed, indicated that α_2-adrenergic pain modulation is activated during inflammation. Brandt and Livingston (1990) showed that during inflammation, the density of α_2-adrenoceptors is increased in laminae I and II of the spinal cord. Also, the turnover of noradrenaline in the spinal cord (Weil-Fugazza et al., 1986) and the activity of the bulbospinal noradrenergic system (Godefroy et al., 1987) increases following experimental arthritis. Our finding that the severity of the inflammatory hyperalgesia to mechanical and thermal stimuli was similar in α_{2A}-adrenoceptor knockout and wild-type was surprising, since the α_{2A}-adrenoceptor subtype mediates the antinociceptive effects of exogenous α_2-adrenoceptor agonists and the endogenous agonist noradrenaline (Hunter et al., 1997; Lakhlani et al., 1997; Stone et al., 1997). It is possible that other α_2-adrenoceptor subtypes are more important in the modulation of inflammatory hyperalgesia. However, the α_{2B}-adrenoceptor is not a likely candidate for pain modulation since it shows mainly peripheral expression (kidney, liver, lung, heart and vascular tissues). The main candidate would then be the α_{2C}-adrenoceptor subtype which is expressed in dorsal root ganglion neurons (Shi et al., 2000) and in the central nervous system (for reviews, see Hein, 2001; Kable et al., 2000; MacDonald et al., 1997). The α_{2C}-adrenoceptor has been linked to presynaptic regulation of neuronal monoamine release (Hein et al., 1999) and to modulation of complex behaviours (Scheinin et al., 2001). In the spinal cord, α_{2C}-adrenoceptor may be expressed in a subset of spinal interneurons (Stone et al., 1998) and there is data indicating that α_{2C}-adrenoceptors are involved in spinal analgesia (Fairbanks et al., 2002). Recently, Lähdesmäki et al. (2002) showed that lack of α_{2A}-adrenoceptor resulted in increased levels of 3-methoxy-4-hydroxyphenylglycol (MHPG), the main metabolite of noradrenaline, in the central nervous system. This indicates that α_{2A}-adrenoceptor knockout mice have increased release of noradrenaline that would be expected to act on their intact α_{2C}-adrenoceptors to produce modulation of pain responses. Lack of a difference in pain responses between α_{2A}-adrenoceptor knockout and wild-type mice does not support the hypothesis that the α_{2C}-adrenoceptor subtype has a major contribution to the endogenous noradrenergic pain modulation.

The α_{2A}-adrenoceptor subtype contributes to the development of thermal but not mechanical hyperalgesia in neuropathic animals (Kingery et al., 2000). In the present study, the response latencies to radiant heat following inflammation were very short (around one second) making it difficult to detect any differences between the experimental groups. Thus, although the present study indicates that α_{2A}-adrenoceptors do not contribute to mechanical hyperalgesia following paw inflammation, a possible contribution of α_{2A}-adrenoceptor to thermal hyperalgesia in inflammatory conditions still needs to be studied further.

It is possible that compensatory changes occur during mouse development in response to the genetic inactivation of a receptor gene. Knocking out a gene might lead to compensatory changes in the expression of other genes or to non-specific changes affecting the behavior of the animal. Such compensatory adaptation was observed by Qiu et al. (2000) who reported that, following inflammation, μ-opioid receptor-deficient mice developed enhanced δ-opioid receptor-mediated antinociception. However, no significant up-regulation of other α_2-adrenoceptor subtypes has been observed in the central nervous system of α_{2A}-adrenoceptor knockout mice (Fagerholm and Scheinin, submitted for publication). Also, the similar antinociceptive effects to morphine in knockout and wild-type animals suggest that there is no compensatory change in the μ-opioid receptor system in the α_{2A}-adrenoceptor knockout mice. Different mouse strains have different sensitivity to noxious stimuli (Lariviere et al., 2001; Mogil et al., 1999) and a mixed

genetic background might confound the effects of a specific genetic manipulation. However, to minimize the effects of a mixed genetic background, the α_{2A}-adrenoceptor knockout mice were backcrossed for five generations to C57Bl/6J mice to form a congenic strain. In the present study, mechanical and thermal withdrawal responses were similar before and following carrageenan-induced inflammation in wild-type and α_{2A}-adrenoceptor knockout mice, which suggests that the sensitivity to noxious stimuli was not affected by the deletion of the α_{2A}-adrenoceptor gene.

4.2. The interaction of μ-opioid receptors and α_{2A}-adrenoceptors in acute nociception and inflammatory hyperalgesia

The results of the present study indicate that α_{2A}-adrenoceptors are not required for the antinociception induced by the μ-opioid receptor agonist morphine in tests of acute nociception and inflammatory hyperalgesia. Previous studies have indicated that μ-opioid receptor and non-subtype-selective α_2-adrenoceptor agonists have synergistic antinociceptive interactions (Ossipov et al., 1989; Sullivan et al., 1987; Wilcox et al., 1987). Earlier studies have also demonstrated that the antinociceptive effects of morphine are attenuated by α_2-adrenoceptor antagonists in models of acute and inflammatory pain (Browning et al., 1982; Herrero and Solano, 1999; Hylden et al., 1991; Ossipov et al., 1989). Therefore, our finding that the absence of α_{2A}-adrenoceptors did not influence morphine analgesia was unexpected. Stone et al. (1997) reported that α_{2A}-adrenoceptors are necessary for spinal adrenergic–opioid synergy. The potency of spinally administered morphine to block pain-like behavior induced by intrathecal substance P was decreased in mice with an inactivating point mutation (D79N) of the α_{2A}-adrenoceptor. However, the potency of morphine was not changed in the tail flick test in the mutant animals. The authors explain that this difference might be due to variation in the capability of different noxious stimuli to activate descending noradrenergic pain modulation that enhances morphine analgesia (Stone et al., 1997). Very short-lasting thermal stimuli used in the tail flick test would not be sufficient to trigger the descending pain suppression, whereas longer lasting pain stimuli, such as intrathecal substance P, would activate the descending noradrenergic neurons and thus enhance morphine analgesia. The results of our study do not support this explanation since no difference was detected in the severity of the hyperalgesia or the responses to morphine during the 24-h follow-up period of carrageenan-induced inflammation. In our study, morphine analgesia was evaluated as mechanically evoked withdrawal responses. However, it should be noted here that, despite various modifications of the testing conditions, we were unable to perform the radiant heat test after morphine administration because of the potent locomotor-activating effects of morphine in mice. Therefore, the conclusion on the lack of interaction between the α_2-adrenoceptors and μ-opioid receptors is based on data only from the mechanical testing with von Frey filaments. A possibility still exists that morphine has different effects on nociceptive responses evoked by radiant heat. Different mechanisms are involved in the different submodalities of pain, which may be one factor contributing to the differences in the results (Treede et al., 1992). In addition, our experiments were performed using mice with targeted inactivation of the gene for the α_{2A}-adrenoceptor. Stone et al. (1997) used α_{2A}-D79N mice, which have a dysfunctional α_{2A}-adrenoceptor protein. Indeed, previous studies have indicated that the locomotor inhibiting or sedative effect of an α_2-adrenoceptor agonist, dexmedetomidine, are different between the α_{2A}-adrenoceptor knockout and α_{2A}-D79N mice (Hunter et al., 1997; Lähdesmäki et al., 2002). Therefore, it cannot be ruled out that the differences between the results of the two studies were, in fact, due to different functional consequences of the type of α_{2A}-adrenoceptor mutation. Also other α_2-adrenoceptor subtypes than α_{2A} may mediate spinal adrenergic–opioid synergy. Fairbanks et al. (2002) showed that the α_{2C}-adrenoceptor subtype is also contributing to the synergy with opioids.

The present study indicates that the absence of α_{2A}-adrenoceptors does not alter the mechanical or thermal hyperalgesia induced by carrageenan, suggesting that α_{2A}-adrenoceptors are not tonically involved in the modulation of inflammatory pain. In addition, μ-opioid receptor agonist-mediated antinociception in acute pain or inflammatory hyperalgesia is not affected by the absence of α_{2A}-adrenoceptors.

Acknowledgements

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