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NEUROSYSTEMS

Tolerance to non-opioid analgesics in PAG involves unresponsiveness of medullary pain-modulating neurons in male rats

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Abstract

Opiate analgesia can be hampered by a reduction in pharmacological effectiveness (tolerance), and this crucially depends on the periaqueductal gray matter (PAG). Non-opioids like metamizol (dipyrone) or aspirin also induce PAG-dependent analgesia and tolerance, but the neuronal bases of this tolerance are unknown. Metamizol is a pyrazolon derivative and cyclooxygenase inhibitor with widespread use as an analgesic in Europe and Latin America. Metamizol was microinjected into the PAG of awake male rats, and antinociception was assessed by the tail flick (TF) and hot plate (HP) tests. Microinjection twice daily for 2.5 days caused tolerance to metamizol. The rats were then anesthetized and recordings from pain-facilitating on-cells and pain-inhibiting off-cells of the rostral ventromedial medulla (RVM) were performed. PAG microinjection of morphine or metamizol depresses on-cells, activates off-cells and thus inhibits nociception, including TF and HP. In metamizol-tolerant rats, however, PAG microinjection of metamizol failed to affect on- or off-cells, and this is interpreted as the reason for tolerance. In metamizol-tolerant rats morphine microinjection into PAG also failed to affect RVM neurons or nociception (cross-tolerance). In naïve, non-tolerant rats the antinociceptive effect of PAG-microinjected metamizol or morphine was blocked when CTOP, a μ-opioid antagonist, was previously microinjected into the same PAG site. These results emphasize a close relationship between opioid and non-opioid analgesic mechanisms in the PAG and show that, like morphine, tolerance to metamizol involves a failure of on- and off-cells to, respectively, disfacilitate and inhibit nociception. Cross-tolerance between non-opioid and opioid analgesics should be important in the clinical setting.

Introduction

Repeated treatment with opiates, the most potent analgesics available, may be hampered by the development of tolerance, i.e. a loss of their antinociceptive effect, and by the risk of withdrawal. To a great extent, the analgesic effect of opiates is due to their action upon the periaqueductal gray matter (PAG; Fields, 2004), and this action is also critical for the development of tolerance (Lane *et al.*, 2005). On the other hand, the action of non-opioid analgesics such as metamizol (dipyrone) and lysine-acetylsalicylate (LASA) is also due in great measure to their effect upon the PAG (Carlsson *et al.*, 1986; Tortorici & Vanegas, 1994, 1995; Vanegas & Tortorici, 2007). The antinociceptive effect of non-opioid analgesics can be reversed by systemic or intra-PAG administration of naloxone (Tortorici *et al.*, 1996; Pernia-Andrade *et al.*, 2004), thus suggesting that non-opioid analgesics share with opioids some common mechanisms of action in the PAG. This suggestion is strengthened by the finding that, like morphine, repeated administration of non-opioid analgesics, either systemically or directly into the PAG, leads to tolerance to their antinociceptive effect, cross-tolerance to morphine and the risk of a withdrawal syndrome (Tortorici & Vanegas, 2000; Pernia-Andrade *et al.*, 2004; Tortorici *et al.*, 2004; Vanegas & Tortorici, 2007).

The PAG exerts its antinociceptive functions largely through neurons located in the rostral ventromedial medulla (RVM). Two classes of RVM neuron have been shown to project to the spinal dorsal horn (Vanegas *et al.*, 1984; Fields *et al.*, 1995) and facilitate (the on-cells) or inhibit (the off-cells) nociceptive transmission (Fields, 2004). Morphine microinjection into the PAG inhibits on-cells and activates off-cells, and this is thought to, respectively, disfacilitate and inhibit nociception at the spinal level (Fields, 2004). When morphine is repeatedly microinjected into the PAG and tolerance thus develops (Jacquet & Lajtha, 1976; Siuciak & Advokat, 1987; Tortorici *et al.*, 1999) further morphine microinjections no longer inhibit on-cells or activate off-cells (Tortorici *et al.*, 2001); this is interpreted as the reason why antinociception fails to occur.

The purpose of the present study was to investigate if, as in the case of morphine, tolerance to PAG metamizol can be explained by a lack of effect upon RVM on- and off-cells. Also, because the antinociceptive action of morphine at the PAG is mostly mediated by μ-opioid receptors (Bodnar *et al.*, 1988; Smith *et al.*, 1988; Fang *et al.*, 1989; Rossi *et al.*, 1994), we investigated if μ-opioid receptors are involved

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in an interaction between opioid and non-opioid analgesics at the PAG. The results showed: (i) that on- and off-cells are indeed involved in metamizol tolerance; and (ii) that PAG microinjection of CTOP, a selective μ-opioid antagonist (Gulya *et al.*, 1988; Hawkins *et al.*, 1989), blocks the analgesic effect of metamizol microinjected into the same PAG site. CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2) is a cyclic analog of the neuropeptide somatostatin and is known to block the antinociceptive effect of morphine (Gulya *et al.*, 1988). Some preliminary results have been presented (Acevedo *et al.*, 2002).

Materials and methods

Ethical guidelines of the International Association for the Study of Pain were followed throughout. This study was approved by the Bioethical Committee for Animal Research of *Instituto Venezolano de Investigaciones Cientificas (IVIC)*. Male Sprague–Dawley rats (bred at IVIC) under thiopental anesthesia (60 mg/kg i.p.) were stereotaxically implanted with a 26-gage chronic guide cannula with the tip located 2 mm above the PAG. During the following week no experiments were carried out but the rats were handled daily in the testing room.

Experiment 1 was designed to investigate the role of on- and off-cells in the tolerance to PAG-microinjected metamizol. Tolerance was induced by microinjecting metamizol (*Novalcina*, a gift from the former Hoechst de Venezuela) into the PAG twice daily for 2.5 days. Metamizol is an antipyretic and analgesic with widespread clinical use in Europe and Latin America (IASP, 1992; Ceraso, 1994; Hoechst, 1996). This pyrazolon derivative readily forms neutral solutions in water and inhibits cyclooxygenase activity (Campos *et al.*, 1999; Chandrasekharan *et al.*, 2002). The pharmacologically active metabolites of metamizol quickly enter the cerebrospinal fluid and reach a concentration in brain tissue of about 50% plasma concentration (Christ *et al.*, 1973; Ochs *et al.*, 1985; Hoechst, 1996; Cohen *et al.*, 1998). In the present study, 1 week after cannula implantation a 33-gage microinjection cannula was introduced into the guide cannula of completely awake rats to reach the ventrolateral PAG, metamizol (150 μg in 0.5 μL saline) was injected over a period of 40 s, and 20 s later the cannula was slowly withdrawn. Nociception was assessed 20 min after microinjection by means of the tail flick (TF) and hot plate (HP) tests. For TF, a light beam was focused on the dorsal surface of the tail to achieve a holding temperature of 35°C. For the actual test this was rapidly increased to 50°C, and the latency to flick was measured (tail flick model 33, IITC, USA); cut-off was 10 s. For HP, the rat was placed on a metal plate at 50°C (hot plate model 39D, IITC) and the latency to first hind paw lick or to jump was measured; cut-off was 30 s. For the induction of tolerance, metamizol microinjections as well as TF and HP testing were carried out at about 09.00 and 15.00 h for 2.5 days. A similar protocol was followed for a control (non-tolerant) group that received only saline microinjections (0.5 μL) into the PAG.

On day 4 metamizol- or saline-treated rats were anesthetized with thiopenthal (60 mg/kg i.p.). Each animal was placed in the stereotaxic apparatus in order to record from RVM on- and off-cells with tungsten microelectrodes (9–12 MΩ; FHC, Bowdoinham, ME, USA). When the initial anesthesia began to lose depth, a diluted solution of thiopental (1/10 of the original dose) was given through a jugular catheter at a constant rate (3.5 mg/mL at 0.016 mL/h). This guaranteed that the TF occured at stable latencies and yet prevented any other manifestation of pain and discomfort. TF latency was recorded every 5 min. On-cells were characterized by their sudden increase in firing, and off-cells by their sudden decrease, just before

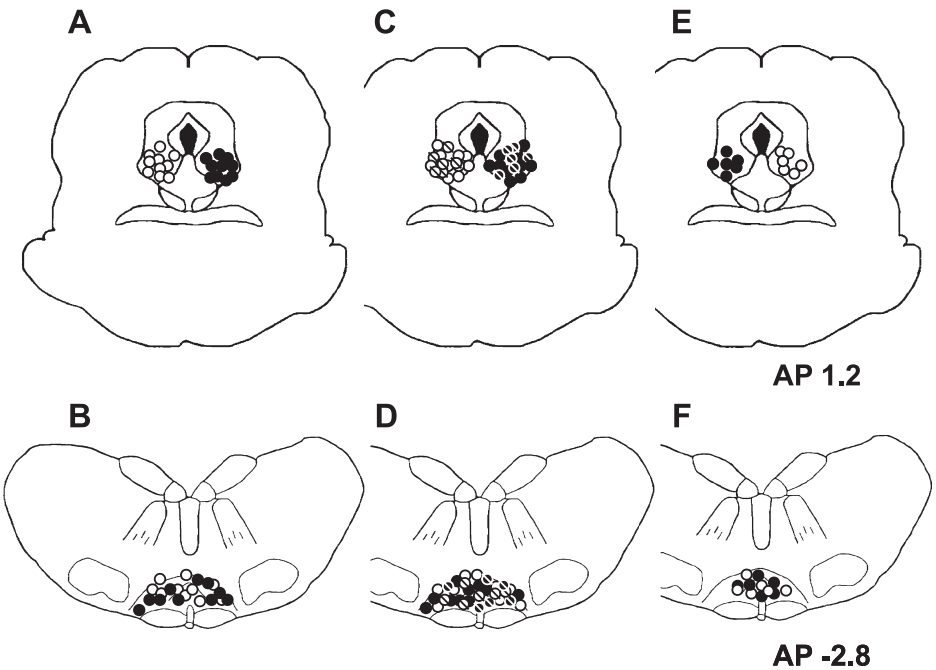

FIG. 1. Histologically verified microinjection sites for metamizol or saline in the PAG (above), and recording sites for on- and off-cells in the RVM (below), in transverse sections simplified from Paxinos & Watson (1998). Experiments with on-cells are depicted with white circles, and experiments with off-cells are depicted with black circles; these are separated in PAG only for clarity as microinjections were done on either side. (A and B) Rats were microinjected with metamizol into the PAG for 2.5 days to induce tolerance. On day 4, RVM on- or off-cells were recorded from and a test microinjection of metamizol was made into the PAG. (C and D) Rats were microinjected with saline into the PAG for 2.5 days. On day 4, RVM on- or off-cells were recorded from and two microinjections of saline (plain circles) or a microinjection of saline followed 20 min later by metamizol (cross-hatched circles) was made into the PAG. (E and F) Rats were microinjected with metamizol into the PAG for 2.5 days. On day 4, RVM on- or off-cells were recorded from, a test microinjection of metamizol was made into the PAG and, 60 min later, a test microinjection of morphine was made into the same PAG site.

TF (Fields, 2004). On- and off-cell activity (spikes/s) was registered within the 2 s that preceded TF or, if absent, cut-off (DataWave Systems, Longmont, CO, USA). Only one on- or off-cell was recorded per rat. In 20 rats that had received metamizol microinjections during the behavioral studies and were therefore metamizol-tolerant, one on-cell ($n = 10$) or off-cell ($n = 10$) was recorded from and metamizol was microinjected into the same PAG site (cf. Fig. 1A and B). In 18 rats that had received only saline microinjections and were therefore non-tolerant (cf. Fig. 1C and D), one on-cell ($n = 9$) or one off-cell ($n = 9$) was recorded from. Saline was microinjected into the same PAG site and, 20 min later, a similar saline microinjection was performed in order to control for the effect of volume injection. In another group of non-tolerant rats ($n = 12$) a similar microinjection of saline was followed 20 min later by a test microinjection of metamizol into the same PAG site (cf. Fig. 1C and D) while recording from on-cells ($n = 6$) or off-cells ($n = 6$). In a further 12 metamizol-tolerant rats, on-cells ($n = 6$) or off-cells ($n = 6$) were recorded from, metamizol was first microinjected into the PAG and, 60 min later, morphine sulfate (generic, 5 μg in 0.5 μL saline) was microinjected into the same PAG site (cf. Fig. 1E and F). PAG microinjection of 5 μg/0.5 μL morphine is known to inhibit on-cells, activate off-cells and inhibit TF (Tortorici & Morgan, 2002). Pilot experiments in naïve, non-tolerant rats had shown that the first, metamizol microinjection (150 μg/0.5 μL) induced an inhibition of TF accompanied by activation of off-cells (three rats) and inhibition of on-cells (three rats). These effects had returned to baseline by 60 min, when the morphine microinjection (5 μg/0.5 μL) was made and caused similar effects.

Experiment 2 was designed to investigate if μ-opioid receptors in the PAG are involved in the antinociceptive action of PAG-microinjected metamizol in awake rats; a positive control was carried out with PAG microinjection of morphine. The guide cannula implantation and the microinjection procedure were like in Experiment 1, but no protocol for repeated microinjections was carried out. Four types of solutions were microinjected into the PAG: metamizol (150 μg/0.5 μL), CTOP (Sigma; 100 ng in 0.5 μL saline), morphine sulfate (5 μg/0.5 μL saline) and saline (0.5 μL). The 5 μg/0.5 μL morphine dose was chosen after preliminary experiments (two rats each) with doses of 1, 2.5, 5 and 10 μg/0.5 μL showed that this dose has an antinociceptive effect comparable to that of metamizol. Also, preliminary microinjections into the PAG (two rats each) demonstrated that 100 ng/0.5 μL CTOP was the minimum dose that prevented the analgesic effect of 5 μg/0.5 μL morphine; CTOP doses of 25 and 50 ng/0.5 μL were ineffective (not shown). The effective CTOP dose used here is comparable to doses used in other microinjection experiments (Shippenberg & Bals-Kubik, 1995; Nobre *et al.*, 2000). For Experiment 2 each rat received two microinjections into the same PAG site, with a 5-min interval between microinjections. Rats in each of four groups (six rats per group) were tested only once with one of the following sequences: saline–morphine; CTOP–morphine; saline–metamizol; and CTOP–metamizol. Nociception was assessed 20 min after the second microinjection by means of the TF and HP tests.

At the end of each experiment the rat was killed with pentobarbital. Cresyl violet (0.4 μL; Sigma, St Louis, MO, USA) was microinjected through the microinjection cannula. An electrolytic lesion was made at the microelectrode tip in Experiment 1. Histological verification (Paxinos & Watson, 1998) of microinjection and recording sites was made on frozen 50-μm transverse sections of the formalin-fixed brain.

The Kolmogorov–Smirnov and the Levene Median tests were applied to verify normality. Thereafter differences between groups were evaluated by unpaired *t*-test or by one-way ANOVA. When appropriate, Bonferroni's multiple comparison test was used to evaluate differences between specific treatments. Statistical significance was acknowledged if $P < 0.05$. The statistical program utilized was GRAPHPAD PRISM, version 3.02 (GraphPad Software, CA, USA).

Results

Experiment 1

Metamizol microinjection (32 rats; Fig. 1A and E) into the PAG on day 1, AM, caused an increase in latency of both TF and HP (Fig. 2). Subsequent metamizol microinjections caused progressively less antinociception, so that by day 2, AM, there was no effect, similar to saline microinjections (30 rats; Figs 1C and 2). In fact, one-way ANOVA revealed significant differences between the latencies detected with both tests ($F_{3,72} = 217.6$, $P \leq 0.0001$ for TF and $F_{3,72} = 759.6$, $P \leq 0.0001$ for HP). The Bonferroni test detected TF

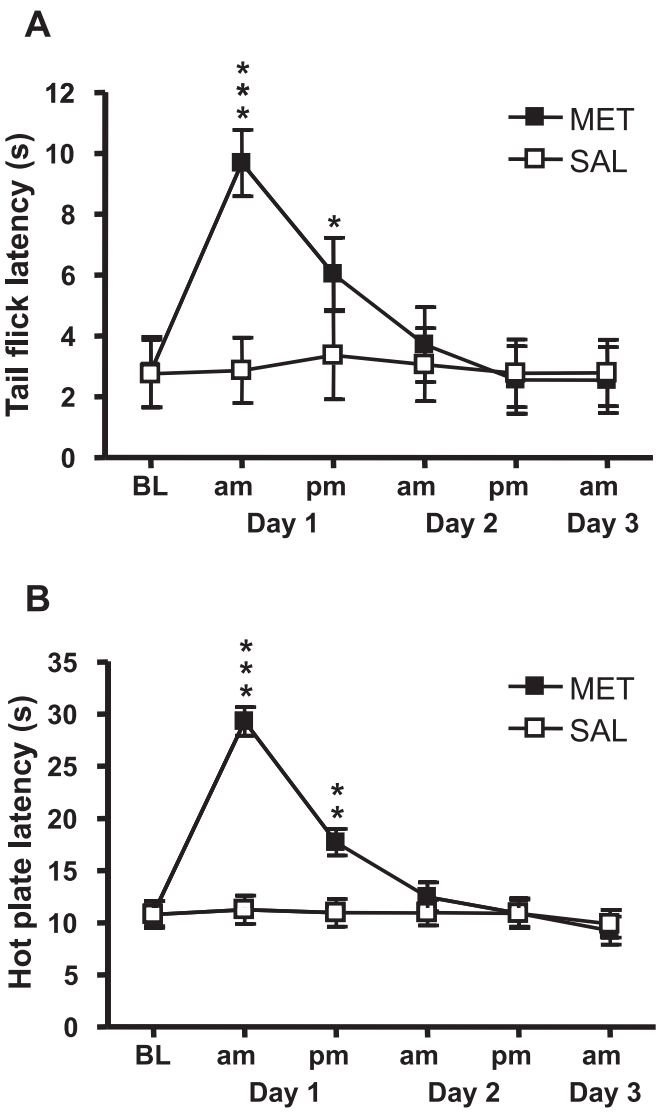

FIG. 2. Development of tolerance to PAG-microinjected metamizol (MET). Antinociception was assessed by the tail flick (TF; A) and the hot plate (HP; B) tests. After baseline (BL) measurements, either MET or saline (SAL) was microinjected into the PAG in the morning (AM) and afternoon (PM) for 2.5 days. Each point represents mean ± SEM. $*P \leq 0.05$, $**P \leq 0.01$ and $***P \leq 0.001$ vs. BL.

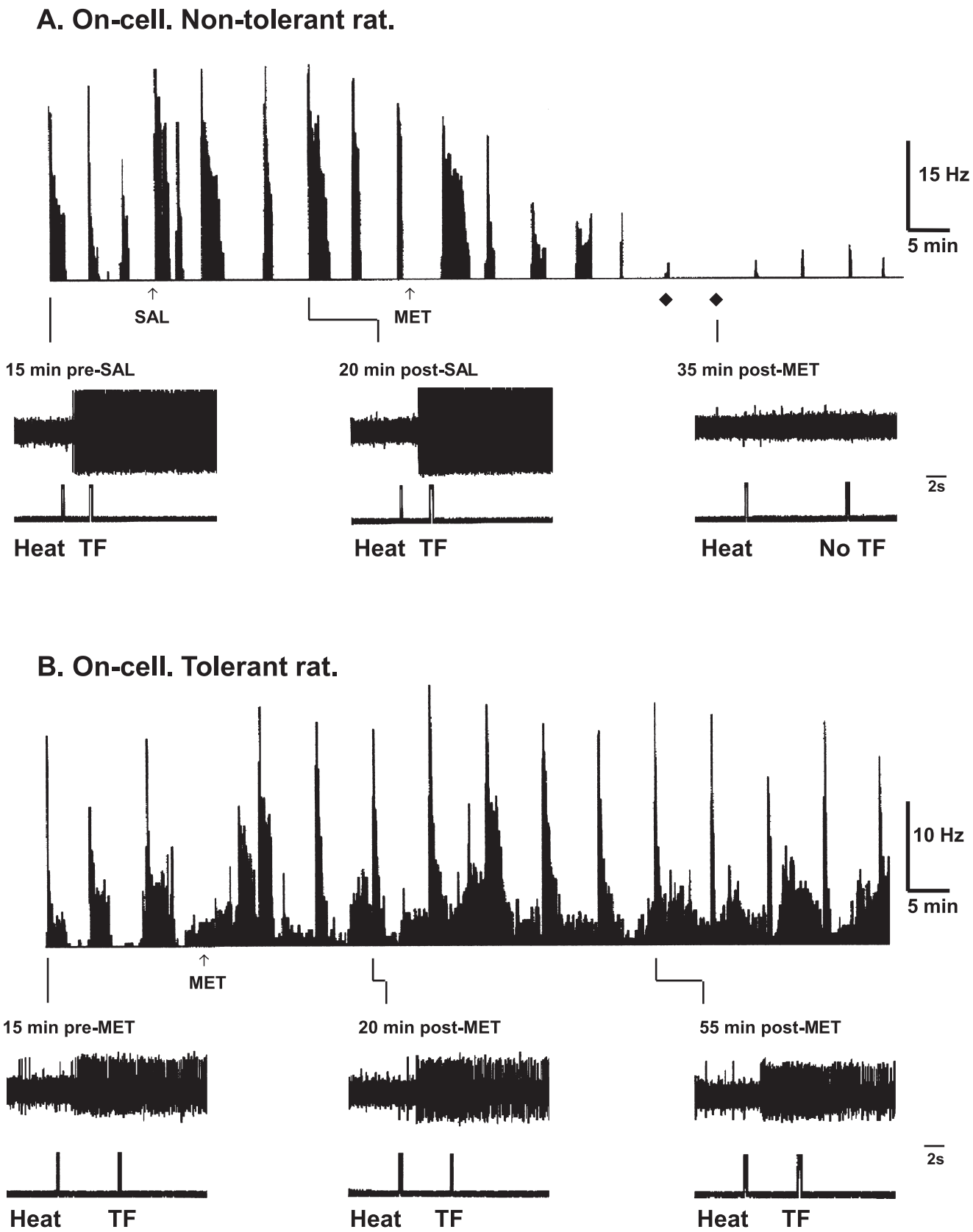

FIG. 3. On-going and heat-elicited activity of RVM on-cells in non-tolerant and metamizol-tolerant rats. (A) An on-cell in a non-tolerant rat. The ratemeter recording (above) shows bursts of activity every time noxious heat is applied to the tail. Selected oscilloscope sweeps (20 s) are shown below; markers indicate the beginning of tail heat and the occurrence of a tail flick reflex (TF) or cut-off (No TF). Saline (SAL) was microinjected into the PAG and had no effect. Then metamizol (MET) was microinjected into the same PAG site. This caused a decrease and eventual loss of the on-cell response and of the TF (rhomboids). (B) An on-cell in a MET-tolerant rat. Same type of display as in (A). Microinjection of MET into PAG had no effect.

differences between metamizol (MET) and saline (SAL) only at early time points [$t = 23.14$, $P \leq 0.001$, day 1 (AM), and $t = 3.610$, $P \leq 0.05$, day 1 (PM)], and also HP differences between MET and SAL only at early time points [$t = 40.28$, $P \leq 0.001$, day 1 (AM), and $t = 15.55$, $P \leq 0.01$, day 1 (PM)]. This confirms previous results on the development of tolerance to PAG-microinjected metamizol (Tortorici & Vanegas, 2000; Tortorici *et al.*, 2004).

Recordings from RVM on-cells were made in 15 rats that had received PAG microinjections of saline for 2.5 days (Fig. 1C and D) and were therefore non-tolerant. On-cells were characterized (Fields, 2004) by their sudden increase in firing before TF occurs during application of heat to the tail (Fig. 3A and B). This was not altered by a test microinjection of saline into the PAG ($n = 15$; Fig. 3A). In contrast (see also Tortorici & Vanegas, 1994), a test microinjection of metamizol into the PAG of non-tolerant rats ($n = 6$; Fig. 1C and D) induced a significant decrease in heat-elicited activity as measured in the last 2 s before TF or cut-off (Fig. 4A; unpaired *t*-test for the activity after SAL vs. activity starting 10 min after MET administration: $t = 42.92$, df = 14, $P \leq 0.001$, two-tailed), as well as a progressively longer delay in the on-cell burst and TF until both failed to occur before cut-off (Fig. 3A). According to well-supported concepts (Fields, 2004), this result suggests that metamizol triggers antinociceptive impulses from PAG, which reduce the excitability of on-cells and prevent them from firing upon heat application; on-cells therefore fail to facilitate spinal nociceptive mechanisms and thus TF fails to occur. In metamizol-tolerant rats (Fig. 1A and B), however, a test microinjection of metamizol into the PAG did not alter the heat-elicited firing of on-cells ($n = 10$; Fig. 4B) and thus TF occurred as usual (Fig. 3B).

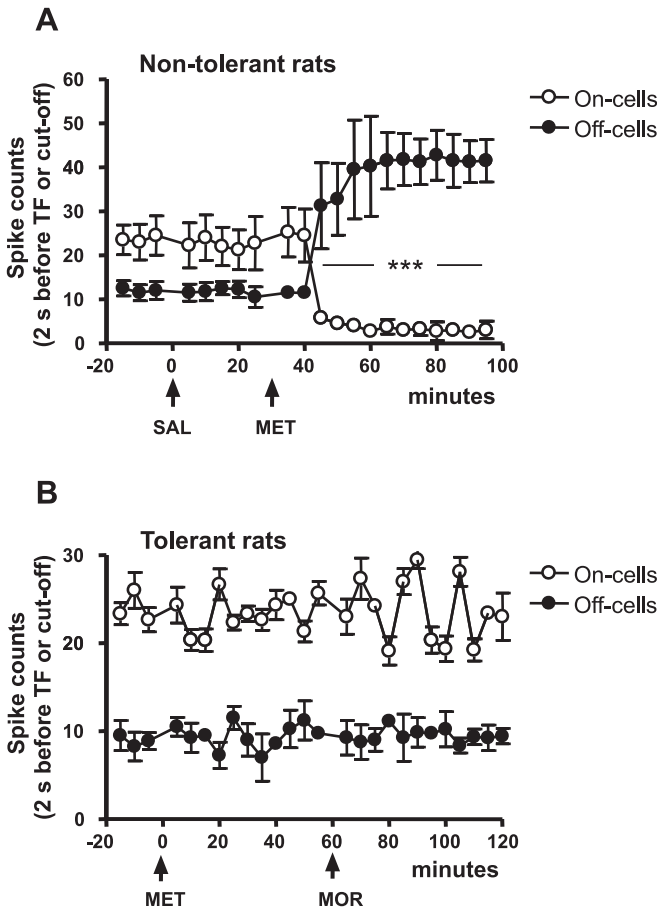

FIG. 4. Activity of RVM on- and off-cells in the 2 s preceding the heat-elicited tail flick reflex (TF) or cut-off (mean ± SEM). (A) In non-tolerant rats, microinjection of saline (SAL) into PAG had no effect, but microinjection of metamizol (MET) into PAG caused a decrease in on-cell firing and an increase in off-cell firing. ***$P \leq 0.001$, starting 10 min after MET microinjection for each cell type vs. post-SAL administration. (B) In MET-tolerant rats neither a microinjection of MET nor a microinjection of morphine (MOR) into the same PAG site caused any significant effect.

Recordings from RVM off-cells were made in 15 rats that had received PAG microinjections of saline for 2.5 days and were therefore non-tolerant. Off-cells were characterized (Fields, 2004) by their sudden decrease in firing before TF occurs during application of heat to the tail (Fig. 5A). This was not altered by a test microinjection of saline into the PAG ($n = 15$; Fig. 5A). In contrast (see also Tortorici & Vanegas, 1994), a test microinjection of metamizol into the PAG of non-tolerant rats ($n = 6$; Fig. 1C and D) induced a significant increase in the activity of off-cells (unpaired *t*-test for the activity after SAL vs. activity starting 10 min after MET administration: $t = 9.382$, df = 14, $P \leq 0.001$, two-tailed), as measured in the last 2 s before TF or cut-off (Fig. 4A), and a progressively longer delay in the off-cell pause and TF until both failed to occur before cut-off (Fig. 5A). Again according to well-supported concepts, this result suggests that metamizol triggers antinociceptive impulses from PAG, which facilitate the activity of off-cells and prevent them from reducing their firing upon heat application; off-cells therefore increase their inhibition of spinal nociceptive mechanisms and thus TF fails to occur. In metamizol-tolerant rats, however, a test microinjection of metamizol into the PAG neither increased on-going activity of off-cells ($n = 10$; Figs 4B and 5B) nor prevented their heat-elicited pause, and thus TF occurred as usual (Fig. 5B).

Finally, in 12 metamizol-tolerant rats, metamizol was microinjected into the PAG (Fig. 1E and F) to show a lack of effect (Fig. 4B). Sixty minutes later a test microinjection of morphine (5 μg/0.5 μL) was given into the same PAG site. Morphine had no effect upon TF or TF-related firing by on-cells ($n = 6$) or off-cells ($n = 6$; Fig. 4B). As far as RVM on- and off-cells are concerned, therefore, tolerance to PAG metamizol was accompanied by cross-tolerance to morphine.

Experiment 2

Because μ-opioid receptors are known to mediate the analgesic effect of PAG-microinjected morphine (Bodnar *et al.*, 1988; Smith *et al.*, 1988; Fang *et al.*, 1989; Rossi *et al.*, 1994), the role of these receptors in the effect of metamizol was investigated. Metamizol microinjections into the PAG were thus preceded by a microinjection of either CTOP ($n = 6$) or saline ($n = 6$) into the same PAG site (Fig. 6). As a positive control, similar experiments were done with morphine. In these tests, one-way ANOVA revealed significant differences between the values detected with both behavioral assays ($F = 423.1$, $P \leq 0.0001$ for TF and $F = 349.5$, $P \leq 0.0001$ for HP). The Bonferroni test detected differences in TF latencies [baseline (BL) vs. 20 min] after using SAL–morphine (MOR): $t = 31.49$, $P \leq 0.001$, or SAL–MET: $t = 29.33$, $P \leq 0.001$. Differences in the HP latencies were also detected (BL vs. 20 min) after using SAL–MOR: $t = 30.12$, $P \leq 0.001$, or SAL–MET: $t = 27.51$, $P \leq 0.001$. However, there was no difference between BL and the 20-min value when either MET or MOR were preceded by CTOP. This means that a concomitant action of endogenous μ-opioid agonists is necessary for the antinociceptive effect of PAG-microinjected metamizol.

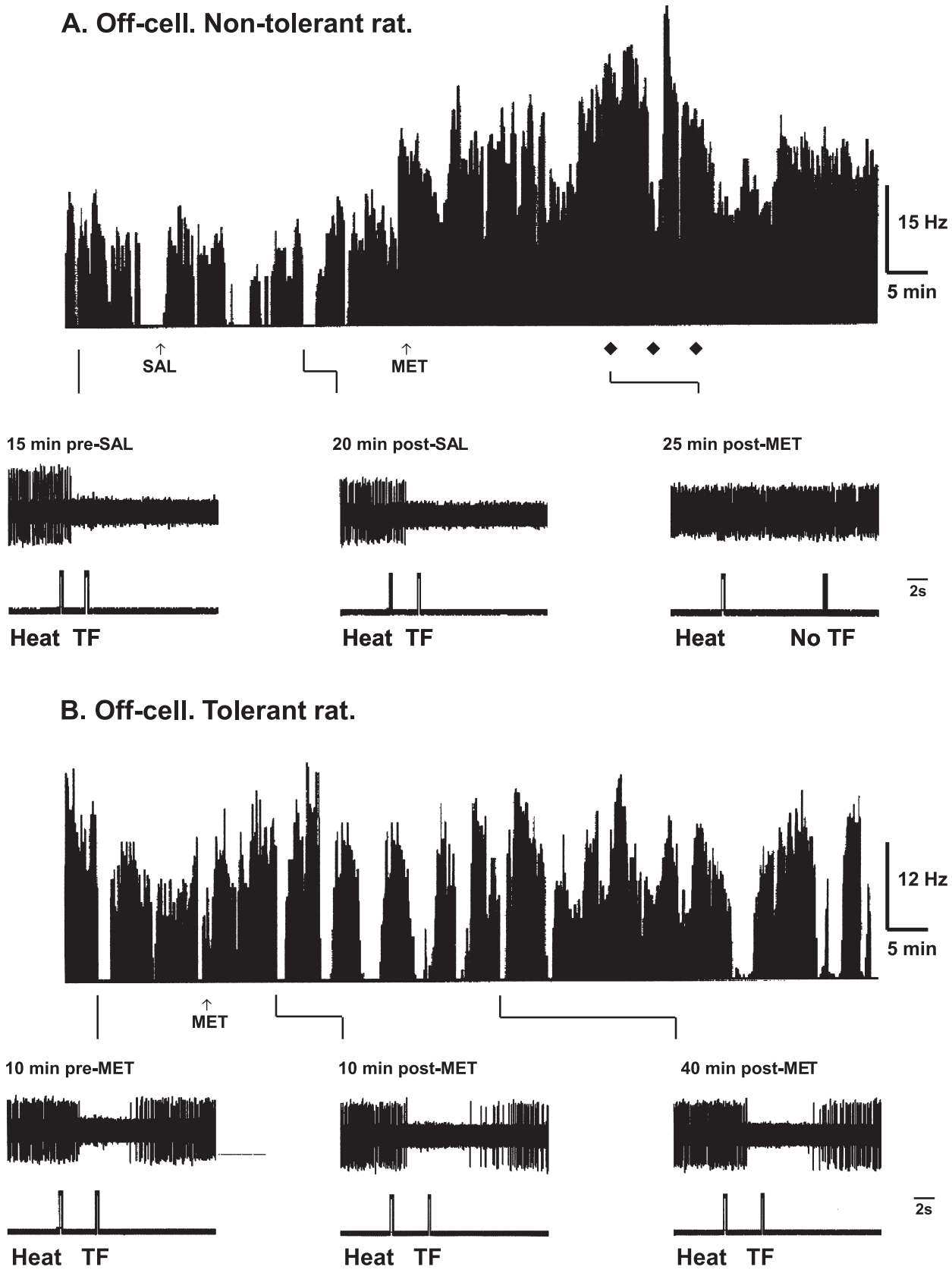

FIG. 5. On-going and heat-elicited activity of RVM off-cells in non-tolerant and metamizol-tolerant rats. (A) An off-cell in a non-tolerant rat. The ratemeter recording (above) shows pauses of activity every time noxious heat is applied to the tail. Selected oscilloscope sweeps (20 s) are shown below; markers indicate the beginning of tail heat and the occurrence of a tail flick reflex (TF) or cut-off (No TF). Saline (SAL) was microinjected into the PAG and had no effect. Then metamizol (MET) was microinjected into the same PAG site. This caused a marked increase in off-cell ongoing activity (and decrease in spike height) and abolished its pause and the TF (rhomboids). (B) An off-cell in a MET-tolerant rat. Same type of display as in (A). Microinjection of MET into PAG had no effect.

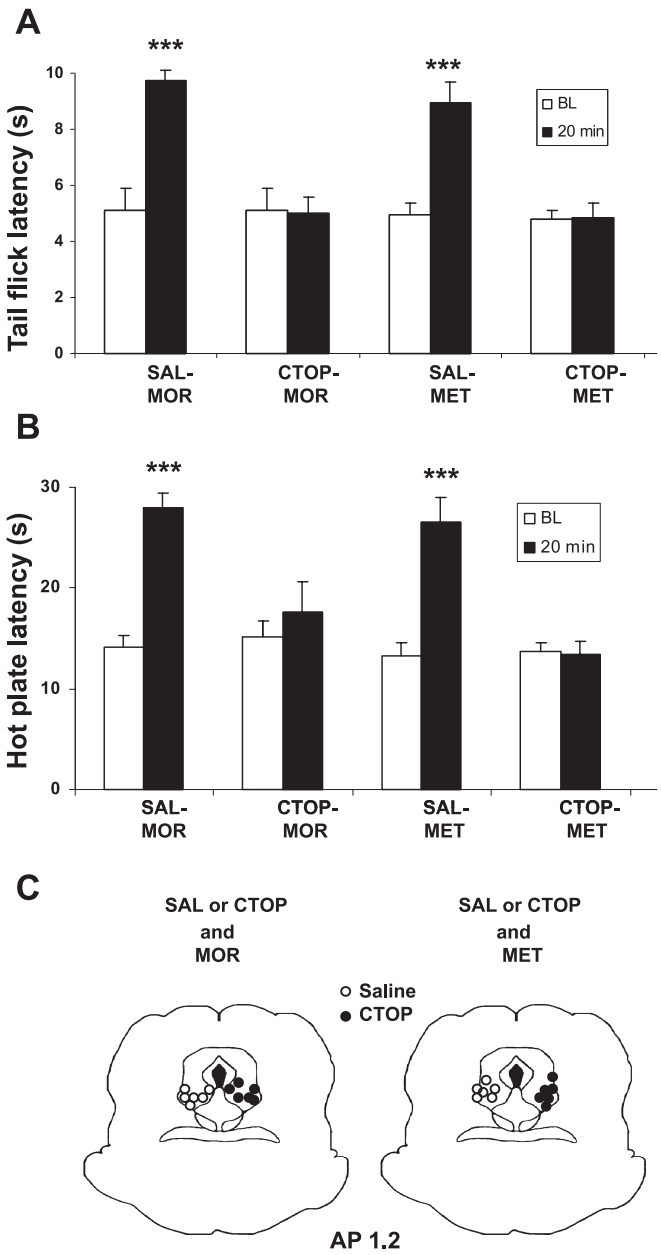

FIG. 6. Prevention of the analgesic effect of a PAG microinjection of either metamizol (MET) or morphine (MOR) by a PAG microinjection of CTOP, a μ-opioid receptor antagonist. After baseline (BL) was determined for TF (A) and HP (B), either saline (SAL) or CTOP was microinjected into PAG. Five minutes later either MET or MOR was microinjected into the same PAG site (C). Previous microinjection of CTOP, but not of SAL, prevented the effect of MET, and these results mimic those obtained with MOR. Each point represents mean ± SEM. ***$P \leq 0.001$ vs. BL.

Discussion

The present findings add evidence to the hypothesis that, like opiates, non-opioid analgesics induce tolerance, and also by mechanisms that heavily bear upon the PAG (Tortorici & Vanegas, 2000; Pernia-Andrade *et al.*, 2004; Tortorici *et al.*, 2004; Vanegas & Tortorici, 2007). The PAG and its descending projection to the RVM are essential components of the descending pain-control system. In naive animals, microinjection of morphine into the PAG decreases the activity of RVM pain-facilitating on-cells and increases the activity of pain-inhibiting off-cells thus giving rise to antinociception (Fields, 2004). Although similar effects can be produced by a direct action of morphine upon the RVM (Heinricher *et al.*, 1992), tolerance to morphine is remarkably difficult to obtain by repeated microinjection into the RVM (Morgan *et al.*, 2005). Furthermore, inactivation of the RVM does not prevent the development of tolerance to repeated morphine microinjection into the PAG, and tolerance to systemic morphine does not develop if opioid receptors are blocked in the PAG even if the RVM remains intact (Lane *et al.*, 2005). The PAG is thus crucial for tolerance to morphine. On the other hand, the PAG is also critical for the antinociceptive action of non-opioid analgesics such as metamizol and LASA (Tortorici & Vanegas, 2000; Pernia-Andrade *et al.*, 2004; Tortorici *et al.*, 2004; Vanegas & Tortorici, 2007). When microinjected into the PAG, these non-opioid analgesics cause a decrease in the activity of on-cells, an increase in the activity of off-cells and an inhibition of nociception (herein and Tortorici & Vanegas, 1994, 1995). The present study now shows: (i) that this analgesic action requires the activation of μ-opioid receptors in the PAG, like in the case of morphine (Bodnar *et al.*, 1988; Smith *et al.*, 1988; Fang *et al.*, 1989; Rossi *et al.*, 1994); and (ii) that, like with PAG microinjections of morphine in morphine-tolerant rats (Tortorici *et al.*, 2001), when metamizol is microinjected into the PAG of metamizol-tolerant rats on- and off-cells fail to respond in their usual manner and antinociception thus fails to occur. Finally, rats tolerant to PAG metamizol are also tolerant to PAG morphine. These results are in line with previous findings showing that the antinociceptive effect of non-opioid analgesics in the PAG is related to endogenous opioids (Vanegas & Tortorici, 2002; Pernia-Andrade *et al.*, 2004; Tortorici *et al.*, 2004), and implies that patients under repeated and prolonged treatment with non-opioid analgesics are, like those under opiates, at risk of tolerance, cross-tolerance and withdrawal.

The mechanisms whereby metamizol and LASA, and probably non-steroidal anti-inflammatory drugs (NSAIDs) in general, engage endogenous opioids in the PAG are incompletely known. γ-Aminobutyric acid (GABA)ergic synapses are one plausible point where NSAIDs could converge with opioids. The PAG output neurons that drive antinociception via downstream relays like the RVM are tonically inhibited by local GABAergic synapses (Moreau & Fields, 1986), and opioids reduce presynaptic release of GABA in the PAG. Indeed, activation of μ-opioid receptors in the PAG brings about an elevation of the intracellular concentration of arachidonic acid, which is then utilized by several molecular pathways. One of these pathways leads to the formation of hepoxilins, which increase potassium conductance; this in turn hyperpolarizes the presynaptic GABAergic terminals and decreases GABA release (Vaughan *et al.*, 1997; Vaughan, 1998). Activity of PAG output neurons would thus increase and drive antinociception. Recent findings have revealed various neuronal relationships that could support PAG–RVM–spinal interactions for nociception (Wessendorf *et al.*, 2006; Morgan *et al.*, 2008).

In another molecular pathway the cyclooxygenases and further enzymes transform arachidonic acid into prostaglandins. The NSAIDs, by blocking the cyclooxygenases, would leave more arachidonic acid available for the synthesis of hepoxilins, thus also further decreasing GABA release (Vaughan *et al.*, 1997; Vaughan, 1998). Similarly, this would be a key pathway for the antinociceptive effect of PAG-microinjected NSAIDs. For this pathway to function, however, an activation of μ-opioid receptors seems to be necessary, because CTOP (present study) or naloxone (Tortorici *et al.*, 1996; Pernia-Andrade *et al.*, 2004) block the effect of PAG-microinjected metamizol or LASA. Because there is no evidence that metamizol or LASA directly activate or in some manner interact with μ-opioid receptors (see, e.g.

Vanegas & Schaible, 2001, p. 347; Brunton *et al.*, 2006, p. 550ff, 555ff, 673ff), the action of non-opioids in PAG would require that endogenous μ-opioid agonists are either tonically present or acutely released. Endogenous opioids do not seem to be tonically present in the PAG, at least in antinociceptive concentrations, because PAG-microinjection of naloxone does not lead to changes in on- and off-cell responses or increased nociception (Tortorici *et al.*, 1996). Tonic release at sub-antinociceptive concentrations or release induced by the microinjection of metamizol or LASA would activate μ-opioid receptors and certainly deserve exploration. An additional mechanism for a metamizol-induced inhibition of GABA release in PAG involves the endocannabinoids. Non-opioid analgesics conserve endocannabinoids because they inhibit the cyclooxygenases and these, among other enzymes, metabolize the endocannabinoids (Bisogno *et al.*, 2005; Fowler *et al.*, 2005). Exogenous cannabinoid agonists decrease GABAergic (and glutamatergic) synaptic transmission in the PAG by decreasing presynaptic transmitter release (Vaughan *et al.*, 2000). AM251, an antagonist/inverse agonist of the CB1 cannabinoid receptor, diminishes the antinociceptive effect of metamizol when both are microinjected into the PAG (Vazquez-Rodriguez *et al.*, 2008). These findings therefore suggest that endocannabinoids contribute to the acute antinociceptive effect of PAG-microinjected metamizol, but an interaction with endogenous opioids, a participation in further mechanisms of tolerance (see below) and a mediation of cannabinoid mechanisms by RVM on- and off-cells must be investigated before any relationship with the results of the present study can be proposed.

At any rate, repetitive interplay between metamizol and opioids in the PAG recruits new players, like the 'antiopioid' cholecystokinin (CCK), to the mechanisms of tolerance. Indeed, tolerance to PAG-microinjected morphine (Watkins *et al.*, 1984; Tortorici *et al.*, 2003) or metamizol (Tortorici *et al.*, 2004) can be prevented or reversed by microinjection of proglumide, a CCK receptor antagonist, into the same PAG site.

These findings underscore the strong convergence of antinociceptive mechanisms of opioids and non-opioid analgesics in the PAG and the role of RVM on- and off-cells in the acute effect of and the development of tolerance to both types of analgesic. The present results also warn against unwanted consequences of such convergence in the clinical setting.

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Abbreviations

CCK, cholecystokinin; CTOP, D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2; GABA, γ-aminobutyric acid; HP, hot plate test; LASA, lysine-acetylsalicylate; NSAID, non-steroidal anti-inflammatory drugs; PAG, periaqueductal gray matter; RVM, rostral ventromedial medulla; TF, tail flick test.

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