Enhancement of $\mu$-Opioid Receptor Desensitization by Nitric Oxide in Rat Locus Coeruleus Neurons: Involvement of Reactive Oxygen Species

J. Llorente, M. T. Santamarta, G. Henderson, and J. Pineda

Department of Pharmacology, Faculty of Medicine and Odontology, University of the Basque Country (UPV/EHU), Bizkaia, Spain (J.L., M.T.S., J.P.); and School of Physiology and Pharmacology, Medical Sciences Building, University of Bristol, Bristol, United Kingdom (G.H.)

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ABSTRACT

It has previously been shown that nitric oxide (NO) synthase is involved in the development of opioid tolerance. The aim of the present work was to study the effect of NO on $\mu$-opioid receptor (MOR) desensitization. Furthermore, we explored the possible role of reactive oxygen species (ROS) in this effect. Single-unit extracellular and whole-cell patch-clamp recordings were performed on locus coeruleus (LC) neurons from rat brain slices. Perfusion with high concentrations of Met5-enkephalin (ME) caused a concentration-related reduction of opioid effect, reflecting the induction of homologous MOR desensitization. The NO donors sodium nitroprusside and diethylamine NONOate markedly enhanced the ME-induced MOR desensitization, although the acute effect of ME on $K^+$ conductance was not affected by sodium nitroprusside. Continuous perfusion with the antioxidants melatonin, trolox, 21-[4-(2,6-di-1-pyrrolidinyl-4-pyrrimidinyl)-1-piperazinyl]-pregna-1,4,9(11)-triene-3,20-dione (U74389G), and diethyldithiocarbamate prevented the effect of sodium nitroprusside on MOR desensitization, but they did not themselves alter the desensitization. Like sodium nitroprusside, the ROS-generating molecule H$_2$O$_2$ enhanced MOR desensitization induced by ME. However, $\alpha_2$-adrenoceptor desensitization induced by noradrenaline was not modified by H$_2$O$_2$, suggesting a selective action of ROS on MOR. Our results suggest that elevated levels of NO, which may be reached in pathological processes, enhance homologous desensitization of MOR in the LC, probably through a mechanism involving ROS generation.

Introduction

Opiates are efficacious analgesics clinically used for pain relief. Long-term or acute high-dose administration of opiates, however, leads to the development of tolerance and dependence, which compromises the clinical efficacy of these drugs. Despite the extensive research conducted, the cellular mechanisms underlying opioid tolerance remain poorly understood. The analgesic effects of opiates occur mainly through the activation of $\Gamma_{i\alpha}$ protein-coupled $\mu$-opioid receptors (MORs); therefore, desensitization of MOR is thought to contribute to the mechanism of opioid tolerance (Taylor and Fleming, 2001). Classic processes of opioid desensitization include G protein-coupled receptor kinase-mediated phosphorylation and internalization of MOR. In addition, the nitric oxide (NO) cascade has been proposed to be involved in opioid tolerance (Tayfun Uzbay and Oglesby, 2001). In the brain, NO is produced by the neuronal NO synthase and...
targets the heme group of guanylate cyclase to elevate cGMP concentrations. Accumulating evidence suggests, however, that cGMP-independent, oxidative, and nitrosative reactions can mediate some of the actions of high, sustained concentrations of NO (Davis et al., 2001). These indirect effects involve reactive oxygen species (ROS) and reactive nitrogen species derived from the reaction of NO with O$_2$ or O$_2^-$. The locus coeruleus (LC), which is the major noradrenergic nucleus in the brain, has been widely used to investigate the cellular mechanisms of action of opioids, because it contains a homogeneous population of neurons that express almost exclusively the MOR (Williams and North, 1984). In brain slices, opioid agonists induce a marked inhibition of LC neurons through a G-protein-dependent activation of inwardly rectifying K$^+$ channel (GIRK) conductance (Nestler and Aghajanian, 1997). Prolonged application of opioid agonists desensitizes MOR-mediated responses in the LC in vitro, but some agonists, such as morphine, seem to cause little acute MOR desensitization in the brain slice (Alvarez et al., 2002; Bailey et al., 2004) yet produce tolerance in vivo. Accordingly, chronic administration of morphine in vivo induces tolerance in the LC (Santamarta et al., 2005; Bailey et al., 2009), which means that certain signaling neuroadaptations occurring in the whole animal trigger MOR tolerance in this nucleus. Proposed candidates for these adaptive mechanisms include cAMP up-regulation (Nestler and Aghajanian, 1997) or PKC activation (Bailey et al., 2004), but the NO signaling pathway may also be involved, because chronic treatment with morphine increases the expression of neuronal NO synthase in the LC (Cueñar et al., 2000). In agreement, inhibition of NO synthase activity attenuates opioid tolerance and withdrawal-precipitated cell hyperactivity in the LC after chronic administrations of morphine in vivo (Highfield and Grant, 1998; Pineda et al., 1998; Santamarta et al., 2005). Furthermore, NO synthase inhibition also prevents the MOR desensitization caused by opioid perfusion in vitro (Torrecilla et al., 2001).

The aim of this work was to investigate whether NO regulates the induction of opioid tolerance; therefore, we explored the effect of the NO donor sodium nitroprusside (SNP) on the desensitization of opioid responses induced by Met$^\gamma$-enkephalin (ME) in vitro. ME has been shown as a potent and efficacious MOR agonist at activating G protein-coupled signaling (McPherson et al., 2010). The effect of the NO donor diethylamine NONOate (DEA/NO) on desensitization was also studied in vitro to further characterize NO effects. Moreover, we attempted to unmask the signaling pathway triggered by SNP in the LC by testing the effect of several antioxidant agents [melatonin, trolox, 21-[4-(2,6-di-1-pyrrolidinyl-4-pyrrimidinyl)-1-piperazinyl]-prenaga-1,4,9(11)-triene-3,20-dione(Z)-2-butenedioate (U74389G), and sodium diethyldithiocarbamate (DCC)] and the ROS-generating molecule H$_2$O$_2$. The present work suggests that NO enhances ME-induced MOR desensitization in the LC through an oxidative pathway, which is prevented by antioxidants and reproduced by the oxidant agent H$_2$O$_2$.

Materials and Methods

Animals

Experimental procedures were conducted in strict accordance with the University of the Basque Country institutional guidelines for animal use in research (Ethics Committee of the Faculty of Medicine), the UK Animals (Scientific Procedures) Act of 1986, and the University of Bristol ethical review document, the European Community Council Directive (86/609/EEC), and the Declaration of Helsinki. Animals were housed under controlled environmental conditions (22°C and 12-h light/dark cycle) with food and water ad libitum. Every effort was made to minimize animal suffering and use the fewest possible number of animals.

Electrophysiological Recording Procedures in the Locus Coeruleus

Slice Preparation. For extracellular recordings, experiments were performed as described previously (Pineda et al., 1996). In brief, male Sprague-Dawley rats (250–320 g) obtained from Harlan (Barcelona, Spain) were anesthetized with chloral hydrate (400 mg/kg i.p.) and decapitated. The brain was removed, and a block of tissue containing the brainstem was rapidly placed in ice-cold artificial cerebrospinal fluid (aCSF) containing 126 mM NaCl, 3 mM KCl, 1.25 mM NaH$_2$PO$_4$, 10 mM d-glucose, 25 mM NaHCO$_3$, and 2 mM MgSO$_4$. Coronal slices of 500-μm thickness including both the LC and ventromedial hypothalamus were cut and submerged in aCSF at 32°C. LC neurons were visualized by Nomarski optics, and individual cell somata were cleaned by gentle flow of aCSF from the slice preparation chamber, which was continuously perfused with oxygenated aCSF at a flow rate of 1.5 ml min$^{-1}$. Drugs were perfused in the bathing medium by switching to a drug-containing solution using a system of manually controlled three-way valves. This system provided an excellent exchange of drugs in the slice, as tested by the effect of a short application of ME. For whole-cell patch-clamp recordings, male Wistar rats (130–170 g) obtained from B&K (Grimston, UK) were killed by cervical dislocation, and their brains were extracted and rapidly submerged in ice-cold cutting solution containing 126 mM NaCl, 2.5 mM KCl, 1.25 mM NaH$_2$PO$_4$, 7 mM MgCl$_2$, 25 mM NaHCO$_3$, and 2 mM MgSO$_4$. coronal slices of 200 to 250 μm in thickness containing the LC were cut and submerged in aCSF containing 126 mM NaCl, 2.5 mM KCl, 1.2 mM MgCl$_2$, 2.4 mM CaCl$_2$, 12 mM NaH$_2$PO$_4$, 11.1 mM d-glucose, and 21.4 mM NaHCO$_3$ (saturated with 95% O$_2$/5% CO$_2$ at 34°C). The slices were left to equilibrate for at least 1 h before recordings were made.

Extracellular and Patch-Clamp Recordings. Single-unit extracellular recordings of neurons were performed as described previously (Torrecilla et al., 2001). The LC was visually identified as a dark oval area in the upper pons on the lateral border of the central gray and the fourth ventricle, just anterior to the genu of the facial nucleus. Omegadot glass micropipettes were pulled and filled with 50 mS sodium chloride, and their tips were broken back to a diameter of 2 to 5 μm (3–5 MΩ). The extracellular signal from the microelectrode was passed through a high-input impedance amplifier (Axolamp 2A, Axon Instruments, Foster City, CA), and then displayed continuously on an oscilloscope and monitored with an audio-analyzer (Cibertec S.A., Madrid, Spain). Individual (single-unit) neuronal spikes were isolated with a window discriminator, and the firing rate was collected and represented by a computer-based, custom-made program (HFCP: Cibertec S.A.), which generated rate bar histograms in consecutive 10-s bins. Noradrenergic neurons in the LC were identified by the following standard electrophysiological criteria: spontaneous discharging activity with regular rhythm, slow firing rate, and long-lasting biphasic positive-negative waveforms (3–4 ms) (Pineda et al., 1996). Only one neuron was recorded per slice, and only one slice was obtained from each animal. Whole-cell patch-clamp recordings were performed as described previously (Bailey et al., 2004). Slices were submerged in a slice chamber (0.5 ml) and perfused with aCSF at a flow rate of 2.5 to 3 ml min$^{-1}$ at 32 to 33°C. LC neurons were visualized by Nomarski optics, and individual cell somata were cleaned by gentle flow of aCSF from a pipette. Whole-cell voltage-clamp recordings (V_m = –60 mV) were made by using electrodes (3–6 MΩ) filled with 115 mM K-gluconate, 10 mM HEPES, 11 mM EGTA, 2 mM MgCl$_2$, 10 mM NaCl, 2 mM...
In these experiments, a progressive reduction in the K<sub>MOR</sub> activation could be continually recorded. The opioid-evoked performing whole-cell patch-clamp recordings a real-time index of analyzed off-line by using pClamp (Molecular Devices). Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) and tested MOR desensitization once H<sub>2</sub>O<sub>2</sub> had been applied to elevate experiment, 100<sup>−6</sup> perfused for at least 20 min before SNP (1 mM) and, in the presence of ROS in the effect of NO, the antioxidant U74389G (10<sup>−4</sup>/H9262 M) was considered as a MOR desensitization. This desensitizing effect was also confirmed by us—/H9262 induced effect (desensitization: 24 < 10<sup>−4</sup>/H11006 M) before and after, respectively, perusions with the desensitizing concentrations of the agonists (3 or 10 < 10<sup>−5</sup>/H11005 M; 100 < 10<sup>−5</sup>/H11005 M). In the patch-clamp recordings, <i>ΔE</i>post is the K<sup>+</sup> current measured at one time point, and <i>ΔE</i>pre is the maximal peak current (before desensitizing). To confirm statistically the development of desensitization, the effects of the agonists were compared before and after the desensitization by a paired Student’s <i>t</i> test. Moreover, to assess the possible differences in the magnitude of desensitization between groups, we compared the degree of agonist-induced desensitization in the absence (control) or the presence of the drug by one-way ANOVA followed by a post hoc least significant differences procedure (SPSS for Windows, version 14.0; SPSS Inc., Chicago, IL). The level of significance was considered as <i>p</i> = 0.05.

### Reagents

ME was purchased from Bachem (Bodendorf, Germany). The following drugs were obtained from Sigma-Aldrich Quimica S.A. (Madrid, Spain): chlora hydrate, H<sub>2</sub>O<sub>2</sub>, NA, SNP, and DDC. Melatonin, trolox, and U74389G were obtained from Alexis Biochemicals (Lausen, Switzerland). DDA/NO was obtained from Cayman Chemicals (Tallinn, Estonia). All other chemicals were obtained from standard sources and were of the highest purity commercially available. Melatonin and U74389G were first dissolved in ethanol and dimethyl sulfoxide and then diluted in aCSF to reach final concentrations of 0.1% (maximum). DDA/NO was first dissolved in NaOH (25 mM; pH 12) to prevent the spontaneous release of NO and then diluted 100 times in aCSF just before its application. We have previously shown that these concentrations of solvents do not have any effect on the firing rate of LC neurons. Other drugs were dissolved directly in aCSF at known concentrations. The full form of the drug as purchased (base or salt) was used in each case in calculating concentrations.

### Results

Desensitization of ME-Induced Firing Inhibition Is Enhanced by NO Donors. To assess MOR desensitization, the inhibitory effect of ME (0.8 < 10<sup>−5</sup>/H11006 M) was evaluated before and after perfusing a high concentration of ME (3 or 10 < 10<sup>−5</sup>/H11005 M) for 10 min. Five minutes after the end of 10 < 10<sup>−5</sup>/H11005 M ME perfusion, the firing rate of LC neurons had returned to the baseline value, but the inhibitory effect of 0.8 < 10<sup>−5</sup>/H11006 M ME was reduced by 63 ± 2% (<i>n</i> = 6; <i>p</i> < 0.005) of the presensitization ME effect (Fig. 1A), indicating the development of desensitization. Using the same protocol, we found that a lower concentration of ME (3 < 10<sup>−5</sup>/H11006 M) only slightly reduced the ME (0.8 < 10<sup>−5</sup>/H11006 M)-induced effect (desensitization: 24 ± 4%; <i>n</i> = 8; <i>p</i> < 0.005) (Fig. 1B).

Previous work has shown that NO synthase inhibitors attenuate MOR desensitization in LC neurons (Torrecilla et al., 2001). Therefore, we used the lower concentration of ME (3 < 10<sup>−5</sup>/H11006 M) to study whether NO released from the donor SNP causes an enhancement of MOR desensitization in the LC. As described previously (Pineda et al., 1996), LC neurons discharged faster during perusions with SNP (0.3 and 1 < 10<sup>−5</sup>/H11005 M) than in the absence of SNP (firing rates: without SNP, 0.85 ± 0.10 Hz; <i>n</i> = 8; 0.3 < 10<sup>−5</sup>/H11005 M; 1.23 ± 0.09 Hz; <i>n</i> = 5, <i>p</i> < 0.05; 1 < 10<sup>−5</sup>/H11005 M; 1,29 ± 0.23 Hz; <i>n</i> = 6) (Fig. 1). The increase in firing activity reached a plateau within 5 to 10 min, after which the firing rate was stable. Administration of SNP (0.3 or 1 < 10<sup>−5</sup>/H11005 M; ≈10 min) enhanced ME (3 < 10<sup>−5</sup>/H11006 M)-induced desensi-
zation by more than 80% of control (desensitization: in the presence of 0.3 mM SNP, 44 ± 6%, n = 5, p < 0.05; 1 mM SNP, 49 ± 8%, n = 6, p < 0.005; compared with slices not perfused with SNP, see above) (Figs. 1, C and D and 2A). In contrast, perfusion with the inactive SNP analog potassium ferricyanide (1 mM) failed to modify ME (3 μM)-induced desensitization (data not shown). Therefore, SNP enhances MOR desensitization, and this effect may be caused by NO release.

To further examine whether NO itself regulates MOR desensitization, we tested the effect of DEA/NO, a nucleophile complex that spontaneously releases NO in aqueous solution, on the ME-induced desensitization of firing inhibition. As expected, DEA/NO (100 μM) increased the firing rate of LC cells (firing rates: before DEA/NO, 0.57 ± 0.06 Hz; after DEA/NO, 1.16 ± 0.20 Hz; n = 5; p < 0.05). Moreover, perfusion with DEA/NO (100 μM) enhanced ME (3 μM)-induced desensitization by approximately 100% of control (desensitization: in the presence of DEA/NO, 49 ± 5%; n = 5; p < 0.05; compared with control slices, see above) (Fig. 1E). These results confirm that NO is able to enhance MOR desensitization.

The Effect of SNP on MOR Desensitization Is Blocked by Antioxidants. NO can react with oxygen derivatives to yield reactive nitrogen derivatives and ROS (Davis et al., 2001). Therefore, to investigate whether ROS production may mediate the enhancement of MOR desensitization induced by NO, LC neurons were recorded during exposure to various antioxidants in the absence or the presence of 1 mM SNP. First, the firing rate, the effect of 0.8 μM ME (1 min), and the desensitization induced by 3 μM ME (10 min) were evaluated in the absence of SNP. During antioxidant perfusions (100 μM melatonin, 200 μM trolox, 10 μM U74389G, or 10 μM DDC) firing rates of LC neurons (0.73 ± 0.09 Hz, n = 5; 0.67 ± 0.15 Hz, n = 5; 0.63 ± 0.12 Hz, n = 5; 0.51 ± 0.04 Hz, n = 5, respectively) and the effects of ME (0.8 μM) (>95%) were not different from those in the absence of the antioxidants. Moreover, perfusion with these antioxidants did not significantly affect the magnitude of ME-induced desensitization (25 ± 6%, n = 5; 21 ± 7%, n = 5; 33 ± 3%, n = 5; and 17 ± 9%, n = 5, respectively) in comparison with the corresponding control group not perfused with any antioxidant (see above) (Fig. 2).

Next, the effect of antioxidants on SNP (1 mM) enhancement of ME (3 μM; 10 min)-induced desensitization was explored. In these experiments, antioxidants were perfused for at least 20 min before SNP application and throughout the rest of the experiment. Perfusion with the antioxidants (100 μM melatonin, 200 μM trolox, 0.1–10 μM U74389G, or 10–100 μM DDC) reduced the enhancement of MOR desensitization induced by SNP. Thus, ME-induced desensitization was approximately 40 to 60% smaller in the presence of the antioxidants than in their absence (desensitization: 1 mM SNP + 100 μM SNP, 25 ± 6%, n = 6, p < 0.005; 1 mM SNP + 200 μM trolox, 31 ± 5%, n = 8, p < 0.05; 1 mM SNP + 10 μM U74389G, 31 ± 6%, n = 6, p < 0.05; 1 mM SNP + 100 μM DDC, 21 ± 5%, n = 5, p < 0.005; versus 1 mM SNP alone in all cases, see above) (Fig. 2). Moreover, the degree of desensitization achieved in the presence of SNP + antioxidants (1 mM SNP + 100 μM melatonin; 1 mM SNP + 200 μM trolox; 1 mM SNP + 10 μM U74389G; 1 mM SNP + 100 μM DDC) was not different from the corresponding value in the presence of the antioxidants alone (100 μM melatonin; 200 μM trolox; 10 μM U74389G; 100 μM DDC; see above) (Fig. 2). This suggests that antioxidants are able to fully prevent the enhancing effect of SNP on MOR desensitization in the LC. Lower concentrations...
of U74389G or DDC did not cause significant reductions in the SNP (1 mM)-induced effect (desensitization: 1 mM SNP + 0.1 μM U74389G, 35 ± 7%, n = 7; 1 mM SNP + 1 μM U74389G, 33 ± 7%, n = 5; 1 mM SNP + 10 μM DDC, 40 ± 3%, n = 5; nonsignificant versus 1 mM SNP) (Fig. 2, B and C). However, when a lower concentration of SNP (0.3 mM) was used to enhance MOR desensitization, the concentration-effect curve for DDC (0.01–100 μM) was shifted to the left with respect to the curve in the presence of SNP (1 mM), and the reduction of SNP (0.3 mM) effect by DDC (10 μM) was then significant (desensitization: 0.3 mM SNP + 10 μM DDC, 26 ± 6%, n = 5, p < 0.05 versus SNP 0.3 mM, see above) (Fig. 2C). This means that the effect of the antioxidants is related to the concentration of both the antioxidant and the NO donor.

**Desensitization of ME-Induced K⁺ Current is Enhanced by SNP in a ROS-Dependent Manner.** To directly confirm the regulation of MOR by NO and ROS in the LC, we measured the K⁺ current induced by ME by using whole-cell patch-clamp recordings in brain slices, which allows a real-time index of MOR activation and desensitization. In neurons clamped at −60 mV, ME (3 μM) evoked a peak outward current of 215 ± 24 pA (n = 5), which desensitized by 30.4 ± 1.8% over the course of a 10-min application (current after 10 min: 150 ± 20 pA; n = 5) (Fig. 3Ai). As with extracellular recordings, 5 min after the end of ME (3 μM) perfusion, the outward current induced by ME (0.8 μM) was reduced with respect to the predesensitization effect (Fig. 3Aii). These results are similar to previously reported data (Harris and Williams, 1991). During perfusion with SNP (1 mM; ≥10 min), the peak current evoked by ME (3 μM) was not altered (outward current: 203 ± 28 pA; n = 4), but the ME (3 μM)-induced desensitization of current responses over the 10-min application was enhanced by a 59% of the control desensitization (without SNP) (desensitization, after 1 mM SNP, 48.2 ± 5.8%, n = 4, p < 0.05 versus control) (Fig. 3A, Ai, Bi, and C). Likewise, SNP (1 mM) enhanced ME (3 μM)-induced desensitization of the ME (0.8 μM) effect (Fig. 3Aiii). It is noteworthy that an application of NA (100 μM) at the end of each experiment (after washout of ME) induced an outward current in the presence of SNP (152 ± 28 pA; n = 4) that was not different from control (without SNP) (160 ± 19 pA; n = 5) (data not shown). This suggests that SNP may not directly affect the GIRK.

As in the extracellular recording studies, we then explored the effect of the antioxidant U74389G perfused for at least 20 min before SNP application and with SNP throughout the rest of the experiment. U74389G (10 μM) prevented the SNP (1 mM)-induced enhancement of MOR desensitization. Thus, the ME (3 μM; 10 min)-induced desensitization of current responses was 44% smaller in the presence of SNP (1 mM) + U74389G (10 μM) than in the absence of the antioxidant (1 mM SNP alone; see above) (desensitization, 1 mM SNP + 10 μM U74389G, 26.8 ± 0.7%, n = 3, p < 0.05 versus 1 mM SNP (Fig. 3, Bi and C). Indeed, the desensitization observed in the presence of SNP + U74389G was not different from control or that in the presence of U74389G (10 μM) alone (Fig. 3, B i-iv and C). U74389G (10 μM) perfusion alone...
affected neither the peak current effect of ME (3 μM) nor the ME (3 μM)-induced desensitization of current responses (desensitization, 10 μM U74389G, 36.1 ± 3.8%, n = 3) (Fig. 3, Biv and C). These data confirm by direct measure of membrane currents that SNP enhances MOR desensitization by a mechanism that is lost during antioxidant perfusion.

**ME-Induced MOR Desensitization Is Enhanced by H$_2$O$_2$.**

To directly examine whether an increase of ROS concentrations may affect MOR desensitization in the LC, we used H$_2$O$_2$ (0.1–1.5 mM), a membrane-permeable ROS-generating molecule that has been reported to be active in brain neurons (Pellmar et al., 1994). ME (3 μM; 10 min)-induced desensitization was tested after perfusion with H$_2$O$_2$ (0.1–1.5 mM for at least 30 min). The firing rates were not significantly changed by any of the H$_2$O$_2$ concentrations (changes from the basal firing rate: 0.1 mM, 0 ± 1%, n = 5; 0.5 mM, −8 ± 10%, n = 5; 1.5 mM, 12 ± 7%, n = 7). Under these conditions, H$_2$O$_2$ administration (1.5 mM) enhanced by 83% the ME-induced desensitization (desensitization, 1.5 mM H$_2$O$_2$, 44 ± 5%; n = 5; p < 0.005; versus control without H$_2$O$_2$, see above) (Fig. 4A). The increase in ME desensitization induced by lower concentrations of H$_2$O$_2$ (0.1 and 0.5) was smaller and did not reach statistical significance (desensitization: 0.1 mM H$_2$O$_2$, 26 ± 8%, n = 5; 0.5 mM H$_2$O$_2$, 37 ± 8%, n = 5) (Fig. 4A). These results indicate that ROS generation by H$_2$O$_2$ enhances MOR desensitization in the LC. To elucidate whether the effect of H$_2$O$_2$ is specific for MOR desensitization, we examined the possible regulation of another Gi/o-coupled inhibitory receptor present in the LC, the α$_2$-adrenoceptor. In the presence of H$_2$O$_2$ (1.5 mM, for at least 30 min), the degree of desensitization of NA (100 μM) effect induced by a high concentration of NA (100 μM; 10 min) (n = 5) was not different from that in the absence of H$_2$O$_2$ (n = 4) (Fig. 4B), suggesting that ROS generation does not enhance α$_2$-adrenoceptor desensitization.

**Discussion**

The involvement of endogenous NO in opioid tolerance has been suggested by data obtained in experiments using NOS inhibitors (Tayfun Uzbugay and Oglesby, 2001), although little is known about the influence of raised levels of NO on this process. The aim of the present study was to investigate the effect of increasing the concentrations of NO with the donor SNP on opioid desensitization and the possible contribution of ROS-dependent mechanisms to the NO effect in the rat LC. To study the desensitization of MOR under physiological conditions in the intact tissue, without altering the neuron milieu, we used the extracellular recording technique in brain slices. Moreover, whole-cell patch-clamp recordings

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**Fig. 3.** Effect of SNP and U74389G on the ME-induced outward K$^+$ current in rat locus coeruleus neurons in vitro. A, sample current recordings show the effect of 0.8 μM ME before and after the application of 3 μM ME in the control (i) or the presence of 1 mM SNP (ii). B, sample current recordings show the time course of the current evoked by 3 μM ME in the control (i) or in the presence of 1 mM SNP (ii), 1 mM SNP plus 10 μM U74389G (iii) or 10 μM U74389G (iv). Scale bars represent 25 pA and 5 min. C, pooled averaged desensitization data express the time course of the 3 μM ME-induced desensitization in control (■; n = 5) (see Ai and Bi) and treated slices. SNP at 1 mM enhanced ME-induced desensitization (●; n = 4) (see Aii and Bii). The antioxidant U74389 at 10 μM prevented SNP-elicited enhancement (▲; n = 3) (see Biii), whereas it did not change by itself the ME desensitization (○; n = 3) (see Biv). *p < 0.05 compared SNP at the last time point with the respective control value. †, p < 0.05 compared SNP + U74389G at the last time point with the respective value with SNP (ANOVA followed by a post hoc DMS test).
were performed to confirm the main data with a direct measure of cell hyperpolarization mediated by MOR.

NA neurons in the rat LC majorly express μ-type opioid receptors, which mediate the GIRK currents and neuron firing inhibition induced by ME (Williams and North, 1984). ME is a potent MOR agonist that activates G protein-mediated signaling with a high efficacy (McPherson et al., 2010). Moreover, it rapidly washes out after perfusion, so that MOR desensitization can be readily measured by extracellular recordings once the spontaneous firing recovers from the inhibition. In our study, ME (3–10 μM) induced a concentration-related MOR desensitization, with a similar degree of functional response to extracellular recordings as reported by voltammetric techniques in the LC in vivo (Desvignes et al., 1997). By comparing these data, we could estimate that the NO concentrations achieved in the slice from the DEA/NO (100 μM) or SNP (1 mg/mL) solutions in our assays would be approximately 3- to 5-fold higher than those physiologically active within the LC. Therefore, the regulation of MOR desensitization by NO described herein is likely to take place under pathophysiological conditions, when elevated concentrations of NO are achieved after N-methyl-D-aspartate administration in the rat LC (Desvignes et al., 1997) or precipitation of opiate withdrawal in morphine-dependent animals (Cuellar et al., 2000).

In agreement with our results, raising the NO concentration by l-arginine administration enhances morphine-induced MOR tolerance in the rat forebrain in vivo (Heinzen et al., 2005). However, lowering the NO concentration by NOS inhibitors attenuates ME-induced MOR desensitization in the LC in vitro (Torrecilla et al., 2001). Like the peptide ME, morphine treatment in vivo causes MOR tolerance in LC neurons (Santamarta et al., 2005; Bailey et al., 2009), but morphine in vitro administration hardly affects MOR functionality (Alvarez et al., 2002). Given our results, this discrepancy could be explained by the observation that chronically applied morphine is able to increase in vivo neuronal expression of NOS in the LC (Cuellar et al., 2000). In fact, NOS inhibitor administration prevents the development of
morpheine-induced tolerance in the LC in vivo (Highfield and Grant, 1998; Santamarta et al., 2005).

It has been proposed that ROS functions as a small signaling messenger that alters the redox state of neuronal macromolecules, thereby affecting the normal functioning of synaptic processes (Knapp and Klann, 2002). Indirect effects of NO can be mediated by its reaction with cellular molecules to yield ROS, which in turn causes oxidative stress (Pellmar et al., 1994; Poderoso et al., 1996). We attempted to investigate the implication of ROS in the effect of SNP on MOR desensitization by testing a battery of structurally unrelated antioxidant and ROS scavengers including melatonin, trolox, U74389G, and DDC. Melatonin is a lipid-soluble antioxidant (Noda et al., 1999), whereas trolox is a cell-permeable vitamin E derivative that prevents oxidative stress in rat models (Balogh et al., 2005). U74389G is a lipid-soluble inhibitor of ROS-induced peroxidation (Khalil et al., 1998), and DDC is a potent reductant that inhibits oxidant-induced damage (Liu et al., 1996). In our extracellular recording assays, all of the tested antioxidants were efficacious in preventing the SNP-induced enhancement of MOR desensitization in the LC. The effects of U74389G and DDC were seen only with the highest concentrations of these drugs, and the potency of DDC was increased by lowering the SNP concentration. Furthermore, the prevention by U74389G was directly measured by patch-clamp techniques. The effect of antioxidants is unlikely caused by a lesser release of NO from SNP in their presence, because decomposition of SNP to yield NO in aqueous solution occurs very rapidly under our conditions (Smith and Dasgupta, 2001). Antioxidants did not alter the intrinsic electrophysiological characteristics of neurons such as the spontaneous firing rate, the holding current, or the basal ME effect and ME (3 μM)-induced desensitization. Hence, antioxidants selectively affected the effect of SNP on MOR desensitization, which suggests that ROS production coupled to target oxidation may mediate, at least in part, the enhancement of MOR desensitization by NO. Likewise, previous studies in mice have reported that melatonin and other antioxidant agents reverse or alleviate opiate-caused behavioral tolerance by restraining NO- and ROS-induced stress (Raghavendra and Kulkarni, 2000; Xu et al., 2006).

To confirm the putative modulation of opioid desensitization by ROS in the LC, we evaluated the effect of the membrane-permeable oxidant H2O2. A similar protocol of H2O2 perfusion has been used to induce oxidative stress through ROS generation in brain slices (Avshalumov et al., 2000; Milusheva et al., 2003). This procedure does not affect the basic electrophysiological features of the neurons (Pellmar et al., 1994). In the LC, perfusion with H2O2 enhanced ME-induced desensitization without changing the NA-induced desensitization, which suggests that ROS generation by H2O2 increases MOR desensitization by a specific mechanism that does not affect similar G_{α/ω}-coupled inhibitory receptors in the LC.

The effect of NO on MOR desensitization through ROS generation could be speculatively ascribed to an oxidation of protein residues in LC neurons. Thus, oxidative reactions have been reported to activate Go_{α/ω} proteins (Nishida et al., 2002), inhibit GIRK (Bannister et al., 1999) and protein phosphatases (Salmeen et al., 2003), and activate PKC (Knapp and Klann, 2002). In our work, modulation of Go_{α/ω} proteins or GIRK is unlikely, because SNP failed to change ME-induced GIRK currents. Moreover, neither SNP nor H2O2 altered a receptor similarly coupled to G proteins and GIRK, the α2-adrenoceptor (Aghajanian and Wang, 1987). The contribution of protein phosphatases could also be ruled out, because phosphatase inhibitors hardly affect MOR desensitization in the LC (Osborne and Williams, 1995). Conversely, a role for PKC would be more plausible, because attenuation of morphine tolerance by NOS inhibitors seems to be mediated by a decline of PKC activity (Liu and Anand, 2001). In the LC, PKC-induced phosphorylation enhances MOR desensitization without changing α2-adrenoceptor desensitization (Bailey et al., 2004, 2009). Molecular data have shown that neuronal NO synthase cascade is activated by morphine though MOR, and the resultant NO recruits PKC to the HINT1-RGS17 complex to eventually phosphorylate and desensitize the MOR (Rodríguez-Muñoz et al., 2008). PKC can also be activated by raising the concentrations of ROS (Knapp and Klann, 2002), and opioids can induce a sustained increase in ROS (Koch et al., 2009). Therefore, one could speculate that in the LC NO enhances MOR desensitization through a ROS-dependent activation of PKC. Future assays should test this hypothesis and the possible involvement of other kinases such as c-Jun N-terminal kinase (Meffie et al., 2010).

In conclusion, the present work indicates that NO released from SNP or DEA/NO enhances ME-induced MOR desensitization in the LC. The findings that this effect is prevented by antioxidants and mimicked by H2O2 suggest that the enhancement of MOR desensitization by NO may occur through oxidative reactions induced by ROS generation. This effect seems specific for this G_{α/ω}-coupled receptor. On the basis of previous studies, NO- and ROS-induced enhancement of MOR desensitization could be mediated by an indirect activation, but the exact mechanism has yet to be determined. In addition, elevations of NO and ROS levels have been shown to mediate several pathophysiological conditions such as ischemia, neurodegenerative diseases, and neurotoxicity (Pellmar et al., 1994). Future investigation should explore whether pharmacological protection against NO/ROS elevation prevents opioid tolerance.

**Authorship Contributions**

- **Participated in research design:** Pineda.
- **Conducted experiments:** Llorente and Santamarta.
- **Performed data analysis:** Llorente.
- **Wrote or contributed to the writing of the manuscript:** Llorente, Henderson, and Pineda.

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