Extracellular Signal-Regulated Kinase 1/2 Activation Counteracts Morphine Tolerance in the Periaqueductal Gray of the Rat

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ABSTRACT

Repeated administration of opioids produces long-lasting changes in μ-opioid receptor (MOR) signaling that underlie behavioral changes such as tolerance. Mitogen-activated protein kinase (MAPK) pathways, including MAPK extracellular signal-regulated kinases (ERK1/2), are modulated by opioids and are known to produce long-lasting changes in cell signaling. Thus, we tested the hypothesis that ERK1/2 activation contributes to the development and/or expression of morphine tolerance mediated by the periaqueductal gray (PAG). Changes in phosphorylated ERK1/2 expression were assessed with confocal microscopy and compared to behavioral measures of tolerance to the antinociceptive effects of chronic morphine administration. Repeated microinjection of morphine into the PAG produced tolerance and caused a significant increase in ERK1/2 phosphorylation, an effect not evident with acute morphine microinjections, demonstrating that ERK1/2 activation was a MOR-mediated event. In summary, these studies show that chronic morphine administration alters ERK1/2 signaling and that disruption of ERK1/2 signaling enhances both the development and expression of morphine tolerance. Contrary to expectations, these data indicate that ERK1/2 activation opposes the development of morphine tolerance.

Opioid receptor activation results in both acute and long-term changes in neuronal physiology. Tolerance, defined as a decrease in agonist potency with repeated administration, is an example of a long-term change caused by opioids. Although tolerance to the antinociceptive effect of morphine develops rapidly with repeated opioid administration (Bailey and Connor, 2005), the signaling mechanism underlying the loss of potency has not been elucidated. One problem with identifying the mechanism is that μ-opioid receptor (MOR) signaling is coupled to several downstream effectors, including inhibition of adenyl cyclase, activation of K+ channels, inhibition of Ca2+ channels, and activation of p42/44 mitogen-activated protein kinases (MAPK) (Spencer et al., 1997; Standifer and Pasternak, 1997; Williams et al., 2001). MAPK extracellular signal-regulated kinases (ERK1/2) are prime candidates for the cellular mechanism underlying tolerance because they are known to contribute to synaptic

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ABBREVIATIONS: MOR, μ-opioid receptor; ERK, extracellular signal-regulated kinase; ANOVA, analysis of variance; cAMP, cyclic AMP; CI, confidence intervals; DOR, δ-opioid receptors; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PAG, periaqueductal gray; vPAG, ventrolateral PAG; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(o-aminophenylmercapto)butadiene ethanolate; DMSO, dimethyl sulfoxide; %MPE, maximal possible effect; β-CNA, β-chlornaltrexamine; SL327, α[amino][4-aminophenyl][thio]methylene]-2-(trifluoromethyl)benzeneacetonitrile.
plasticity (Mazzucchelli et al., 2002; Pouyssegur and Lenormand, 2003). ERK1/2 signaling has been implicated in several types of synaptic plasticity, including long-term potentiation, long-term depression, long-term memory formation, including spatial learning and fear-conditioning, and in dendritic spine formation (Sweatt, 2004; Thomas and Huganir, 2004). Previous work demonstrates that MOR agonists may increase and/or decrease the MAPK pathway depending on the time course or system under investigation. For example, there is a lack of ERK1/2 activation following acute morphine treatment of cultured neurons or acute morphine administration in rats (Schulz and Höltt, 1998; Muller and Unterwald, 2004; Macey et al., 2006). These findings suggest that ERK1/2 does not contribute to the actions of acute morphine. However, activation of ERK1/2 has been demonstrated in dorsal root ganglion neurons and several brain regions in mice and rats following chronic morphine treatment (Ortiz et al., 1995; Berhow et al., 1996; Ma et al., 2001; Narita et al., 2002; Asensio et al., 2006), indicating that adaptations during prolonged exposure to morphine may induce MOR coupling to ERK1/2. On the other hand, phosphorylated ERK1/2 was down-regulated in human opiate addicts and rats following chronic morphine treatment (Ferrer-Alcón et al., 2004; Muller and Unterwald, 2004), indicating that in some cases chronic opiate administration is correlated with a decrease in ERK1/2 phosphorylation.

Although opiates act on many sites throughout the nervous system, the descending nociceptive modulatory system that runs from the periaqueductal gray (PAG) to rostral ventromedial medulla to the dorsal horn of the spinal cord seems to be particularly important in both morphine antinociception and tolerance. Microinjection of morphine into the ventrolateral PAG (vlPAG) produces antinociception, and repeated administration produces tolerance (Jacquet and Lajtha, 1976; Siuciak and Advokat, 1987; Tortorici et al., 1999; Lane and Morgan, 2005; Morgan et al., 2006). Furthermore, activation of opioid receptors in the vlPAG blocks the development of tolerance to repeated systemic administration of morphine (Lane and Morgan, 2005). Thus, the vlPAG is an ideal brain site to test whether ERK1/2 activation contributes to morphine tolerance. Given that ERK1/2 is known to contribute to neuronal plasticity, it is hypothesized that repeated administration of morphine into the vlPAG will activate ERK1/2 and that blocking ERK1/2 will also block morphine tolerance.

Materials and Methods

Subjects
Male Sprague-Dawley rats (200–350 g; Animal Technologies, Livemore, CA) were anesthetized with pentobarbital (60 mg/kg i.p.) and implanted with a guide cannula (23 gauge, 9 mm long) aimed at the vlPAG (AP: +1.7 mm, ML: ±0.6 mm, DV: −5.0 mm from lambda) using stereotaxic techniques. The guide cannula was attached to two screws in the skull by dental cement. At the end of the surgery, the rat received a prophylactic injection of the antibiotic cefazolin (15 mg/0.15 ml i.m.), and a stylet was inserted to plug the guide cannula. The rat remained in place an additional 20 s to minimize backflow of the drug up the cannula track. After the injection, the stylet was replaced, and the rat was returned to its home cage.

After surgery, rats were housed individually. The animal housing room was maintained on a reverse light/dark schedule (lights off at 7:00 AM) so rats could be tested during the active dark phase. Food and water were available at all times except during testing. Rats were handled daily before and after surgery. Testing began at least 7 days after surgery. All procedures were conducted in accordance with the Institute of Laboratory Animal Resources (1996). Efforts were made to minimize the number of experimental subjects (e.g., using a within subjects design when possible).

Nociceptive Assessment
Nociception was assessed using the hot-plate test. The hot-plate test consisted of measuring the latency to lick the hind paw when placed on a 52.5°C plate. The rat was removed from the hot plate if no response occurred within 50 s.

Microinjections
Drugs were administered through a 31-gauge injection cannula inserted into and extending 2 mm beyond the tip of the guide cannula. Rats received a sham injection in which an injector was inserted into the guide cannula but no drug was administered. This procedure reduces confounds resulting from mechanical stimulation of neurons on the test day and habituates the rat to the microinjection procedure. Testing with drug administration began 1 day later. Drugs were microinjected in a volume of 0.4 μl and at a rate of 0.1 μl/10 s while the rat was gently restrained by hand. The injection cannula remained in place an additional 20 s to minimize backflow of the drug up the cannula track. After the injection, the stylet was replaced, and the rat was returned to its home cage.

A cumulative microinjection procedure was used to assess changes in morphine antinociceptive potency. Increasing cumulative doses of morphine sulfate (a gift from the National Institute on Drug Abuse, National Institutes of Health, Bethesda, MD) in third log doses of 1, 2, 4, 6, 10, and 22 μg/0.4 μl were injected into the PAG every 20 min. Nociception was assessed using the hot-plate test 15 min after each injection. This procedure has been shown to produce reliable dose-response curves (Morgan et al., 2005, 2006; Meyer et al., 2007).

Experiment 1: Does ERK1/2 Activation Contribute to the Development of Morphine Tolerance?

The development of tolerance to the antinociceptive effect of morphine and activation of ERK1/2 were assessed in morphine tolerant animals pretreated with the MEK inhibitor U0126 (Sigma-Aldrich, St. Louis, MO). Animals received microinjections of vehicle (saline or 20% DMSO) or the MEK inhibitor U0126 (4.7 μM) 20 min before receiving doses of morphine (5 μg/0.4 μl) or saline twice daily on days 1 and 2. Nociception was assessed before and 15 min after microinjection of morphine or saline after the first set of injections on day 1 but not after subsequent injections on days 1 and 2 to avoid confounds from repeated testing (Milne and Gamble, 1989; Lane and Morgan, 2005). U0126 (100 ng) was dissolved in saline or 20% DMSO and sonicated before microinjection. A fresh aliquot was used for each set of injections. The same shift in D₅₀ values was observed whether U0126 was dissolved in saline or 20% DMSO; therefore, groups were collapsed for data analysis. Antinociceptive tolerance was assessed on day 3 using the morphine cumulative dose procedure described above. Animals were exposed to a lethal dose of halothane and perfused with formalin within 5 min after the last hot-plate test. The brain was removed, and PAG slices were prepared for ERK1/2 immunolabeling as described below.

The effect of blocking opioid receptors after tolerance had developed was assessed to determine whether ERK1/2 activation caused by repeated morphine administration was mediated through an opioid receptor-dependent mechanism. Animals received morphine (5 μg/0.4 μl) or saline microinjection twice a day for 2 days. On day 3, animals were injected twice with the irreversible opioid receptor antagonist β-CNA (0.270 μg/0.5 μl) (Sigma-Aldrich) or vehicle (0.1% ethanol) 6 h and 30 min before the morphine cumulative dose procedure as described above. The brain was removed for ERK1/2 immunolabeling as described previously.

Experiment 2: Does ERK1/2 Activation Contribute to the Expression of Morphine Tolerance?

The involvement of ERK1/2 on the expression of tolerance to the antinociceptive effect of mor-
phine was assessed in morphine-tolerant animals treated with the MEK inhibitor U0126. Animals received twice daily microinjections of morphine (5 μg/0.4 μl) or saline on days 1 and 2. Nociception was assessed before and 15 min after microinjection with morphine or saline after the first of injections on day 1. Tolerance was assessed on days 3 and 4. Animals were pretreated with U0126 (4.7 μM) or vehicle 20 min before the morphine cumulative dose procedure, as described above, to determine whether blocking ERK1/2 activation alters the expression of tolerance. A within-subjects design was used with saline and U0126 administered in a counterbalanced manner on days 3 and 4.

Histology
Rats were perfused with saline and then formalin (10% mixed with saline) within 5 min of the last hot-plate test. The brain was removed and placed in formalin (10%). At least 2 days later, the brain was sectioned coronally (60 μm) and the location of the injection site identified (Fig. 1) (Paxinos and Watson, 2005). Brains with correct placements from experiment 1 were processed for ERK1/2 immunolabeling.

Immunohistochemistry
Coronal brain slices containing vlPAG were incubated in 5% goat serum (Sigma-Aldrich), 3% bovine serum albumin (Sigma-Aldrich), and 0.5% Triton X-100 (Sigma-Aldrich) for 1 h at room temperature. All of the slices were then incubated in a cocktail of primary antibodies containing rabbit anti-phospho-ERK1/2 (1/300; Cell Signaling, Beverly, MA) and mouse anti-NeuN (1/500; Chemicon, Temecula, CA) overnight, washed, and then incubated for 2 h in a cocktail of secondary antibodies containing goat anti-rabbit IgG Alexa Fluor 488 (1/800; Invitrogen, Carlsbad, CA) and goat anti-mouse IgG Alexa Fluor 555 (1/800; Invitrogen) conjugates. Slices were washed, mounted onto a slide with SlowFade (Invitrogen), coverslipped, and imaged with a Zeiss LSM510 META confocal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY). The anti-NeuN antibody confirmed that activation of ERK1/2 was specific to neuronal cells.

Phospho-ERK1/2 expression was quantified in individual NeuN-labeled vlPAG neurons adjacent to the aqueduct using the Zeiss LSM Image Examiner program (Carl Zeiss MicroImaging, Inc.) (Fig. 1). Neurons used for analysis were selected based on abundant NeuN labeling in one optical slice through vlPAG. With the phospho-ERK1/2 channel turned off, each NeuN-labeled neuron in the optical slice was traced using the tracing tool in the LSM Image Examiner program. The experimenter was blind to treatments during analysis. A separate box was drawn in an area without labeling to measure background fluorescence for each optical slice per one animal. The phospho-ERK1/2 channel was then turned on, and the absolute frequencies [number of pixels/pixel intensity (0–255)] of phospho-ERK1/2 and background labeling were measured by LSM Image Examiner. The area of each neuron was also measured by LSM Image Examiner in square micrometers. The weighted intensities of phospho-ERK1/2 labeling and background labeling were calculated for each neuron as [sum of (absolute frequency × intensity) (at each intensity)]/square micrometers (Excel; Microsoft, Redmond, WA). The weighted intensity of the background labeling was subtracted from that of the phospho-ERK1/2 labeling to obtain the final intensity/square micrometer of phospho-ERK1/2 labeling for each neuron. The average intensity of the mean (±S.E.M.) for the total number of neurons in each treatment group (n = 2–6 rats/group; 6–11 cells per rat) was then calculated. Statistical significance was determined by an ANOVA followed by Dunnett’s post hoc test, p < 0.05 or p < 0.01 (Prism; GraphPad Software, Inc., La Jolla, CA).

Behavioral Data Analysis.
Hot-plate latencies were converted to maximal possible effect [%MPE = (test latency − baseline)/(50 s − baseline)]. The lower and upper limits for calculating D50 values were set at 0 and 100% MPE, respectively. Morphine dose-response curves and D50 values (dose for half-maximal antinociception) (Taladra, 2001) were calculated using nonlinear regression (Prism; GraphPad Software, Inc.). Ninety-five percent confidence intervals (CI) were used to compare individual D50 values following a significant ANOVA (see “Experiments 1 and 2”). ANOVA also was used to determine whether morphine or U0126 had acute effects, and Tukey’s post hoc test was used to compare specific groups when there was a significant main effect, p < 0.05. Only rats with injection cannula in or on the border of the vlPAG were included in data analysis.

Results

Experiment 1: Does ERK1/2 Activation Contribute to the Development of Morphine Tolerance? Baseline hot-plate latencies measured before injections on day 1 ranged from 12.7 to 15.4 s for the four groups. Microinjection of morphine into the vlPAG caused a significant increase in hot-plate latency compared with saline [F(3,39) = 22.82; p < 0.05; Fig. 2]. The effect of microinjecting morphine or saline was not altered by pretreatment of U0126.

Fig. 1. Region of the vlPAG where microinjections were administered and ERK1/2 phosphorylation was assessed. Rectangles depict the region of the PAG where ERK1/2 activation was assessed using confocal microscopy [Reproduced with permission from Paxinos G and Watson SJ (2005) The Rat Brain in Stereotaxic Coordinates. Academic Press, New York. Copyright © Elsevier, 2005]. Slices imaged were 50 μm away adjacent to the site of injection. Morphine microinjections were in or immediately adjacent to this rectangle.
D50 values for development and expression of morphine tolerance

**Table 1**

<table>
<thead>
<tr>
<th>Morphine Tolerance</th>
<th>D50 (µg)</th>
<th>CI</th>
<th>No. of Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Development</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sal/Sal</td>
<td>4.6</td>
<td>3.3–5.9</td>
<td>9</td>
</tr>
<tr>
<td>U0126/Sal</td>
<td>4.2</td>
<td>3.4–5.0</td>
<td>10</td>
</tr>
<tr>
<td>Sal/MOR</td>
<td>5.8</td>
<td>4.8–6.8</td>
<td>10</td>
</tr>
<tr>
<td>U0126/MOR</td>
<td>9.4</td>
<td>8.3–10.5</td>
<td>14</td>
</tr>
<tr>
<td>Expression</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>6.0</td>
<td>2.2–9.8</td>
<td>8</td>
</tr>
<tr>
<td>U0126</td>
<td>10.8</td>
<td>6.2–15.3</td>
<td>8</td>
</tr>
</tbody>
</table>
In addition, an increase in the levels of phospho-ERK1/2 in the cerebral cortex and striatum was reported following an acute, high dose of morphine (100 mg/kg) injected intraperitoneally in rats but not with lower doses of morphine (10 and 30 mg/kg i.p.) (Asensio et al., 2006). This result is consistent with observations that ERK1/2 activation is increased with acute administration of the more efficacious agonist fentanyl in striatal neurons (Macey et al., 2006) and with the full MOR agonist, [d-Ala², N-Me-Phe⁴,Gly⁵-ol]-enkephalin acetate salt, in MOR-transfected cells (Belcheva et al., 1998), suggesting that ERK1/2 activation by MOR may be preferentially stimulated by high-efficacy agonists.

Our finding that ERK1/2 phosphorylation occurs in rats following repeated morphine administration indicates that activation of MOR by opioid agonists is altered with repeated injections. Increased phosphorylation of ERK1/2 following repeated morphine exposure also has been shown in cultured dorsal root ganglion neurons (Ma et al., 2001) and in other brain regions, including the ventral tegmental area and pons/medulla following chronic, subcutaneous morphine treatment (Ortiz et al., 1995; Behrooz et al., 1996; Narita et al., 2002; Asensio et al., 2006). Although it is possible that an acute, higher dose of morphine may activate ERK1/2 in the vIPAG, our previous data suggest that repeated administration of morphine induces an increase in potency of opioid agonists, as measured by an increase in G protein-coupled inwardly rectifying potassium channel signaling in the vIPAG (Ingram et al., 2008). This enhanced activation of MOR may be responsible for increased ERK1/2 activation following repeated morphine administration and could explain the activation by acute administration of high-efficacy agonists compared with acute administration of morphine.

The regulation of ERK1/2 is a candidate for understanding the cellular mechanism of tolerance due to its role in synaptic plasticity. The activation of ERK1/2 during the development and expression of tolerance is dependent on the tissue, agonist, and time investigated. In this study, blockade of ERK1/2 phosphorylation by the MEK inhibitor U0126 in the vIPAG...
shifts the morphine dose-response curve to the right (enhanced tolerance), suggesting that direct activation of ERK1/2 may oppose tolerance. This is contrary to the prevalent view that ERK1/2 activation with repeated administration of morphine leads to an increase in tolerance but is consistent with the observation that chronic morphine increases MOR potency in the PAG, stimulates phosphorylation of MOR (Schmidt et al., 2000), and increases MOR desensitization (Ingram et al., 2008). Therefore, MOR-mediated activation of ERK1/2 may play a role in the counteradaptations that occur during the development and expression of antinoceptive tolerance.

Although ERK1/2 is known to contribute to other forms of plasticity, the ability of ERK1/2 to counteract morphine tolerance has not been reported previously. In fact, a recent study showed that the intraperitoneal injection of the MEK inhibitor SL327 in mice had no effect on morphine tolerance (Mouledous et al., 2007). The MEK inhibitor U0126 at higher concentrations inhibits ERK5, another MAPK family member that responds to oxidative stress and epidermal growth factor (Mody et al., 2001). However, we did not use a high concentration of U0126 and do not expect that the regulation of ERK5 changed during morphine tolerance. Again, our data demonstrate a direct behavioral correlation of enhanced tolerance following inhibition of MEK signaling.

The increase in ERK1/2 activation following chronic morphine administration correlates with a wide range of other neuronal changes associated with the development of tolerance. Changes within the vPAG include an increase in morphine potency (Ingram et al., 2008), the apparent mobilization of δ-opioid receptors (DOR) to the plasma membrane (Commons et al., 2001; Hack et al., 2005), and increased signaling of second messengers such as cAMP (Ingram et al., 1998; Bagley et al., 1999). The modulation of ERK1/2 via MOR activation by second messengers (Belcheva et al., 2005) suggests the up-regulation of various second messengers, including cAMP, after chronic morphine treatment (Ingram et al., 1998) could be the mechanism by which ERK1/2 activation is induced. An additional pathway that may lead to ERK1/2 activation may include the activation of cAMP through the DOR. Whether DOR and cAMP up-regulation are part of the same or different pathways is unclear, but both changes drive ERK1/2 activation after chronic morphine treatment. For example, the formation of MOR-DOR heterodimers has been shown to activate ERK1/2 (Rozendfeld and Devi, 2007). These results are provocative in that enhancement of ERK1/2 signaling may be a mechanism by which a drug could attenuate tolerance to morphine.

Acknowledgments

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