ATP-sensitive Potassium Channels and L-type Calcium Channels are Involved in Morphine-induced Hyperalgesia after Nociceptive Sensitization in Mice

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**A B S T R A C T**

**Introduction:** We investigated the role of ATP-sensitive potassium channels and L-type calcium channels in morphine-induced hyperalgesia after nociceptive sensitization.

**Methods:** We used a hotplate apparatus to assess pain behavior in male NMRI mice. Nociceptive sensitization was induced by three days injection of morphine and five days of drug free. On day 9 of the schedule, pain behavior test was performed for evaluating the effects of morphine by itself and along with nimodipine, a blocker of L-type calcium channels and diazoxide, an opener of ATP-sensitive potassium channels. All drugs were injected through an intraperitoneal route.

**Results:** The results showed that morphine (7.5, 10 and 15 mg/kg) induced analgesia in normal mice, which was prevented by naloxone (1 mg/kg). After nociceptive sensitization, analgesic effect of morphine (10 and 15 mg/kg) was significantly decreased in sensitized mice. The results showed that nimodipine (2.5, 5, 10 and 20 mg/kg) had no significant effect on pain behavior test in either normal or sensitized mice. However, nimodipine (20 mg/kg) along with morphine (10 and 15 mg/kg) caused more decrease in morphine analgesia in sensitized mice. Furthermore, diazoxide by itself (0.25, 1, 5 and 20 mg/kg) had also no significant effect on pain behavior in both normal and sensitized mice, but at dose of 20 mg/kg along with morphine (10 and 15 mg/kg) decreased analgesic effect of morphine in sensitized mice.

**Discussion:** It can be concluded that potassium and calcium channels have some roles in decrease of analgesic effect of morphine after nociceptive sensitization induced by pretreatment of morphine.

**Key Words:** Pain Behavior, Sensitization, Morphine, Diazoxide, Nimodipine.

1. **Introduction**

Morphine is among the most effective and commonly used analgesics for controlling moderate to severe pain (Benyamin et al., 2008; Cunha et al., 2010; Somogyi, Barratt & Coller, 2007). It has been shown that antinociceptive effect of morphine is mediated via an inhibitory G protein, which inhibits cAMP formation and calcium (Ca2+) conductance, while activates potassium (K+) conductance that induces hyperpolarization of nociceptive cells (Nestler, 2004; Rodrigues & Duarte, 2000). Therefore, although morphine effects are mediated by mu-opioid receptors but ion channels may play important roles in its effects. In support of this idea, some evidence has indicated that ATP-sensitive potassium (K+ATP) channels are involved in morphine analgesia (Chiou & How, 2001). It has been also shown that morphine, via activation of a signaling pathway including K+ATP channels, blocks...
hypernociception by changes in membrane potential of nociceptive neurons (Cunha et al., 2010). Furthermore, blockade of calcium channels has been also reported to alter analgesic effects of morphine in laboratory animals. In particular, it has been shown that both central and systemic administrations of L-type calcium channel blockers potentiate morphine analgesia (Benedek & Szikszay, 1984; Contreras, Tamayo & Amigo, 1988; Del Pozo, Caro & Baeyens, 1987; Dogrul, Yesilyurt, Isimer & Guzeldemir, 2001; Omote, Sonoda, Kawamata, Iwasaki & Namiki, 1993).

Besides its well-known antinociceptive actions, morphine can cause hyperalgesia by an unknown opioid receptor-independent mechanism (Chu, Angst & Clark, 2008). Opioid–induced hyperalgesia is defined as a state of nociceptive sensitization caused by exposure to opioids, which is related to but different from tolerance (Lee, Silverman, Hansen, Patel & Manchikanti, 2011; Silverman, 2009). Although this effect is constantly reported across literature, the neurochemical changes and mechanisms associated with this phenomenon remain unknown (Chu, Angst & Clark, 2008). Adaptive change in neurons is a hallmark of chronic morphine treatment, and related to altered behaviors associated with morphine dependence (DuPen, Shen & Ersek, 2007). However, the molecular and cellular mechanisms underlying these long-lasting changes are not still fully understood (Lee, Silverman, Hansen, Patel & Manchikanti, 2011; Raffa & Pergolizzi, 2012).

It has been reported that a regimen of 3 days morphine and then 5 days washout may induce sensitization to morphine (Rezayof, Assadpour & Alijanpour, 2013; Zarindast & Rezayof, 2004). We have recently shown that blockades of K+ATP and Ca2+ channels decrease the analgesic effect of morphine in diabetic mice (Ahmadi, Ebrahimi, Oryan & Rafieenia, 2013). According to research, a sensitization process in pain pathways may be involved in diabetic-induced hyperalgesia (Kamei et al., 1994; Voitenko, Kruglikov, Kostyuk & Kostyuk, 2000). Considering these backgrounds, the aim of the present study was to investigate the role of K+ATP and Ca2+ channels in the decrease of analgesic effect of morphine in mice after nociceptive sensitization induced by morphine.

2. Methods

2.1. Subjects

Adult male albino NMRI mice weighing 20-30 g (Pasteur institute, Tehran, Iran) were kept in an animal house with a 12/12-h light/dark cycle (light on at 7:00 a.m.) and controlled temperature (22±2°C). They were housed in groups of 10 in Plexiglas cages with free access to food and water. Pain behavior test was performed during the light phase of the cycle, and each animal was tested once only. All procedures were performed in accordance with guide of National Academy of Sciences’ Institute (NASI) for care and use of laboratory animals (2011).

2.2. Drugs

Morphine sulfate was purchased from Temad (Tehran, Iran). Naloxone hydrochloride, diazoxide, an K+ATP opener, and nimodipine, a blocker of L-type and Ca2+ channels were purchased from Ascent Scientific (Bristol, UK). Morphine and naloxone were dissolved in saline (0.9 %, w/v solution) before each use, while nimodipine and diazoxide were dissolved in a vehicle composed of dimethylsulfoxide (DMSO) and saline (1:1 v/v solution). All drugs were injected through an intraperitoneal route at a volume of 10 ml/kg. Drug doses were selected either from pilot experiments or other studies (Biala & Weglinska, 2006; Sukriti, Hota & Pandhi, 2004).

2.3. Induction of Nociceptive Sensitization in Mice

Nociceptive sensitization schedule was performed during 8 days. First, mice were injected intraperitoneally with morphine (20 mg/kg) for three consecutive days, and then they were allowed to spend five days of drug free (wash out). Control group only received normal saline instead of morphine in the same way. One day after the sensitization schedule (on day 9), the animals were tested for pain behavior on a hotplate apparatus.

2.4. Hotplate Test

A hotplate apparatus (Armaghan Co., Iran), was used to assess algesic or analgesic effects of drugs. On the testing day, the mice were acclimated to the testing environment for 30 min, and then each animal placed on the plate of apparatus which its temperature was set at 55±0.1°C. A glass square (height 25 cm) was placed on the hotplate to prevent escaping of the animal. Pain behavior was defined as either licking of the hind paws or first jumping of the animal. The time elapse from the placement of the animal on the hotplate until observing pain behavior was recorded as baseline or test latencies. A cutoff time of 120 s was defined to avoid tissue damage. “Baseline latency” was measured just prior to drug administration, and “test latency” was recorded after drug treatments at time intervals of 30 min after morphine, 20 min after naloxone or 45 min after nimodipine and diazoxide. Finally, the recorded “baseline latency” and
“test latency” were converted to percent maximum possible effect (%MPE) according to the following formula:

\[
\text{%MPE} = \left(\frac{\text{test latency} - \text{baseline latency}}{\text{cut-off time} - \text{baseline latency}}\right) \times 100.
\]

2.5. Experimental Design

2.5.1. Experiment 1: Effects of Morphine on Pain Behavior Test in Normal and Sensitized Mice

Fifteen groups of animals were used. Ten groups of them received saline during three days followed by five days washout. On hotplate test day, baseline latency was recorded for each animal. Then, test latency was recorded for five groups of these animals 30 min after administrations of saline or morphine (5, 7.5, 10 and 15 mg/kg). The other five groups received saline or morphine (5, 7.5, 10 and 15 mg/kg) at 30 min before testing plus naloxone at 20 min before testing, then test latency was recorded. The last five groups of the animals received pretreatment of morphine (20 mg/kg) for three days followed by five days washout to induce nociceptive sensitization. On the hotplate test day (day 9), baseline latency was firstly recorded, and then test latency was measured at 30 min after administrations of saline or morphine (5, 7.5, 10 and 15 mg/kg).

2.5.2. Experiment 2: Effects of Nimodipine by itself on Pain Behavior Test in Normal and Sensitized Mice

In this experiment, ten groups of mice were used. Five groups of them received pretreatment of morphine (20 mg/kg) for three days, and then they were allowed to spend five days washout to induce nociceptive sensitization. On hotplate test day, baseline latency was firstly recorded, and then test latency was measured 30 min after administrations of saline or morphine (5, 7.5, 10 and 15 mg/kg), and test latency was recorded 45 min after administrations of the drugs.

2.5.3. Experiment 3: Effects of Nimodipine along with Morphine on Pain Behavior Test in Sensitized Mice

Ten groups of animals were submitted to the nociceptive sensitization schedule. On the hotplate test day, baseline latency was measured for each animal. Then, five groups of them received vehicle (10 ml/kg) but the other five groups received nimodipine (20 mg/kg) at 45 min before recording the test latency. Fifteen min after vehicle or nimodipine injections, five groups of both sets received saline or different doses of morphine (5, 7.5, 10 and 15 mg/kg), and test latency was measured 30 min after the last injection.

2.5.4. Experiment 4: Effects of Diazoxide by itself on Pain Behavior Test in Normal and Sensitized Mice

Ten groups of mice were used. Five groups of them received pretreatment of morphine (20 mg/kg) for three days followed by five days washout to induce nociceptive sensitization, but the other five groups as normal mice only received saline. On hotplate test day, baseline latency was firstly recorded, then five groups of either normal or sensitized mice received saline or diamoxide (0.25, 1, 5 and 20 mg/kg), and test latency was recorded 45 min after administrations of the drugs.

2.5.5. Experiment 5: Effects of Diazoxide along with Morphine on Pain Behavior Test in Sensitized Mice

In this experiment, five groups of animals were submitted to nociceptive sensitization schedule. On the hotplate test day, baseline latency was firstly recorded. Then, all groups immediately received diamoxide (20 mg/kg), 15 min later, they received saline or morphine (5, 7.5, 10 and 15 mg/kg), and 30 min after the last injection, the test latency was recorded for each animal. Five groups of mice as control groups of experiment 3, which received vehicle (instead of diamoxide) plus saline or morphine (5, 7.5, 10 and 15 mg/kg) were also considered as control groups for experiment 5.

2.6. Statistical Analysis

All data were presented as mean±S.E.M. of %MPE related to ten animals in each group. One- or two-way analysis of variance (ANOVA) was used for analyzing data. Following a significant F-value, post-hoc t-test was performed to assess paired groups comparisons. P<0.05 was considered as statistically significant level.

3. Results

3.1. Morphine Induced Analgesia in Normal Mice, but its Analgesic Effect was Decreased in Sensitized Mice

The results of experiment 1 showed that morphine induced significant analgesia in normal mice [one-way ANOVA, F (4, 45) = 21.74, P< 0.001]. Post-hoc t-test revealed that morphine at doses of 7.5, 10 and 15 mg/kg induced significant analgesia compared to saline-treated
control group in normal mice. One-way ANOVA also revealed that injections of naloxone along with morphine decreased analgesic effect of the later drug, as revealed by decrease of between-group significance [one-way ANOVA, F (4, 45) = 3.37, P<0.05]. Posthoc t-test revealed that naloxone almost completely prevented the analgesic effect of different doses of morphine except for 15 mg/kg. Two-way ANOVA showed a significant interaction between sensitization schedule (as factor A with two levels) and morphine treatment on hotplate test (as factor B with five levels) [F (4, 90) = 3.29, P<0.05]. However, although sensitization induced a significant decrease in the opioid analgesia compared to normal mice, but its effect was not equal to the blockade of opioid receptors by naloxone (Fig. 1).

3.2. Nimodipine by itself had neither Algesic nor Analgesic Effects in both Normal and Sensitized Mice

One-way ANOVA revealed that nimodipine injection on hotplate test day caused no change in pain behavior test of either normal mice [one-way ANOVA, F (4, 45) = 0.12, P>0.05], or sensitized mice [one-way ANOVA, F (4, 45) = 1.06, P>0.05] (Fig. 2).

3.3. Nimodipine Decreased Analgesic Effect of Morphine in Sensitized Mice

A two-way ANOVA was conducted to explore the effect of nimodipine (factor A with two levels) and morphine (factor B with five levels) on pain behavior test, as measured by %MPE. The interaction effect between nimodipine and morphine was not statistically significant [F (4, 90) = 2.37, P>0.05]. However, there was a statistically significant effect on pain behavior test for nimodipine [F (1, 90) = 23.18, P<0.001], and morphine [F (4, 90) = 24.63, P<0.001]. Analyses of post-hoc t-test showed that analgesic effect of morphine at doses of 10 and 15 mg/kg plus nimodipine (20 mg/kg) was significantly decreased compared to the respective groups that received morphine plus vehicle (Fig. 3).
3.4. Diazoxide by itself had no Significant Effect on Pain Behavior Test in either Normal or Sensitized Mice

One-way ANOVA revealed that diazoxide had neither significant analgesic nor algesic effect in both normal mice [one-way ANOVA, F (4, 45) = 2.58, P>0.05], or sensitized mice [one-way ANOVA, F (4, 45) = 0.4, P>0.05] (Fig. 4).

3.5. Diazoxide Caused more Decrease in Analgesic Effect of Morphine in Sensitized Mice

A two-way ANOVA was conducted to explore the effect of diazoxide (factor A with two levels) and morphine (factor B with five levels) on pain behavior test, as measured by %MPE. There was a statistically significant effect for diazoxide [F (1, 90) = 66.38, P<0.001], and for morphine [F (4, 90) = 23.85, P<0.001]. The interaction effect between diazoxide and morphine was also statistically significant [F (4, 90) = 4.5, P<0.01]. Analyses of post-hoc t-test showed that analgesic effect of morphine at doses of 10 and 15 mg/kg was significantly decreased by co-injections of diazoxide (20 mg/kg) compared to the respective groups that received morphine plus vehicle (Fig. 5).
4. Discussion

The results of the present experiments showed that morphine dose-dependently induced analgesia in normal mice, but its analgesic effect was decreased in mice with pretreatment of 3 days morphine followed by 5 days washout. Although the analgesic effect of morphine is well known, however, there are many reports about decreasing morphine-induced analgesia after chronic use of the drug, which is known as opioid-induced hyperalgesia or nociceptive sensitization induced by the opioid (Mao, Price & Mayer, 1995; Silverman, 2009). The effects of morphine have been shown to be mediated primarily through activation of mu-opioid receptors (Bodnar, 2011). In support of this claim, it has been shown that the analgesic, rewarding, and withdrawal-induced aversive effects of morphine are eliminated in mice disrupted for mu-opioid receptors (Matthes et al., 1996; Sora et al., 1997). The present results also showed that analgesic effect of morphine in normal mice was prevented by coinjections of naloxone along with morphine on hotplate test day, which confirm that analgesic effect of morphine is mediated through mu-opioid receptors.

We used a previously reported regimen in mice receiving pretreatment of 3-days morphine (high concentration) followed by 5-days washout to induce nociceptive sensitization (Rezayof, Assadpour & Ali jampour, 2013; Zarrindast & Rezayof, 2004). According to our results, the above used regimen of morphine induced a nociceptive sensitization as revealed by a decrease of analgesic effect of morphine in sensitized mice, one day after the regimen. This result may also be reported as opioid-induced hyperalgesia after nociceptive sensitization. One possibility for decrease in the analgesic effect of morphine after the nociceptive sensitization in the present study may be due to an increase in pain perception resulted from changes in mu-opioid receptors and related signaling pathways in nociceptive neurons. It has been also reported that opioid-induced hyperalgesia may result from insensitivity to opioids (Garnier et al., 2003). It has been shown that multiple signal transduction pathways including protein kinases, second messengers such as cAMP and ion channels may be engaged once mu-opioid receptors is activated with morphine (Freye & Lataesch, 2003; Koch & Hollt, 2008; Liu & Anand, 2001). Therefore, it is possible that not only changes in mu-opioid receptors but also in its downstream signaling molecules including ion channels may underlie the altered analgesic efficacy of morphine.

According to a research, repeated exposure to drugs of abuse such as morphine also results in locomotor sensitization, which leads to an increase in locomotor response (Valjent et al., 2010). Therefore, an alternate possibility for decrease of analgesic effect of morphine after nociceptive sensitization may result from changes in locomotor activity. However, we did not examine locomotor activity, and the above suggested possibility needs more experiments to be clarified. We have previously reported the involvement of L-type calcium channels (Ca\(^{2+}\) channels) and ATP-sensitive potassium channels (K\(^+\) channels) in hyperalgesia induced by morphine in diabetic mice. Therefore, we hypothesize that these channels may also play a role in the hyperalgesia induced by morphine after nociceptive sensitization.

The results of the present study revealed that nimodipine, a blocker of L-type Ca\(^{2+}\) channels by itself had neither algesic nor analgesic effect in both normal and sensitized mice. The results also showed that the higher dose of nimodipine along with morphine caused more decrease in analgesic effect of morphine in sensitized mice. Several studies reported that administration of Ca\(^{2+}\) channel blockers induce analgesia in laboratory animals (Hrvat, Brodacz & Holzer-Petsche, 2001; Miranda et al., 1992). However, in the present study nimodipine by itself did not induce either algesic or analgesic effect in both normal and sensitized mice. Other studies also reported that nimodipine improves analgesic effect of morphine in normal animals (Kumar, Mehra & Ray, 2010; Michaluk, Karolewicz, Antkiewicz-Michaluk & Vetulani, 1998; Shimizu et al., 2004; Zharkovsky, Katajamaki, Seppala & Ahtee, 1999). In the present study, we administrated nimodipine along with morphine to investigate possible involvement of Ca\(^{2+}\) channels in the decreased analgesic effect of morphine in sensitized mice. An explanation for decreasing the analgesic effects of morphine with nimodipine in sensitized mice may be due to the fact that Ca\(^{2+}\) channels are affected by pretreatment regimen of morphine sensitization. However, some other factors such as different subjects, different tests for examining pain behavior and drug doses may account for the discrepant results of this study with others.

The results of the present study also indicated that activation of K\(^+\)-ATP channels by diazoxide, an opener of K\(^+\)-ATP channel induced neither algesic nor analgesic effects in both normal and sensitized mice. The results also showed that the higher dose of diazoxide along with morphine on hotplate test day caused significant interaction with morphine to decrease the analgesic effect of the later drug in sensitized mice. It has been reported that peripheral analgesic effectiveness of morphine is greatly abrogated during neuropathic pain states (Rashid, Inoue, Matsumoto & Ueda, 2004), and the reduction in K\(^+\)-ATP
currents have been reported for the reduced analgesic effectiveness of morphine (Cunha et al., 2010). Since diazoxide caused more decrease in analgesic effect of morphine, we propose that K+ATP channels in sensitized mice have been influenced by pretreatment of morphine. It has been reported that intracerebroventricular administrations of the K+ATP channel blockers antagonize the analgesic effects of opioids (Ocana, Del Pozo, Barrios & Baeyens, 1995; Ocana, Del Pozo, Barrios, Robles & Baeyens, 1990; Wild, Vanderah, Mosberg & Porreca, 1991). Our results also showed that diazoxide antagonized the analgesic effect of morphine in sensitized mice. Blockade of KATP channels has been proposed to suppress the activation of descending noradrenergic system induced by i.c.v. injections of morphine (Narita et al., 1992). In this study, we examined the effects of K+ATP channels and L-type Ca2+ channels in morphine-induced hyperalgesia in a diabetic mice model (Ahmadi, Ebrahimi, Oryan & Rafieenia, 2013), and it was observed that the blocker of these channels decreased the diabetes-induced hyperalgesia. On the contrary, the present results showed that nimodipine and diazoxide increased the hyperalgesia in sensitized mice, as revealed by a more decrease in analgesic effect of morphine after a regimen of nociceptive sensitization. Comparing these results with our previous study, it can be proposed that there are different mechanisms underlying the hyperalgesia induced by being either diabetic or sensitized mice models.

5. Conclusions

In summary, the analgesic effect of morphine was decreased in sensitized mice. Nimodipine and diazoxide decreased analgesic effect of higher doses of morphine in sensitized mice. It can be concluded that analgesic effect of morphine after sensitization have been influenced by K+ATP channels and Ca2+ channels. Our results propose that potassium and calcium channels may be the potential target points for controlling the pain in patients with chronic use of morphine.

References


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