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Short communication

Inhibition of protein phosphatases alters the expression of morphine tolerance in mice

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Abstract

Recently our laboratory found that tolerance to morphine-induced antinociception could be completely reversed with intracerebroventricular (i.c.v.) administration of a protein kinase A inhibitor, whereas intrathecal (i.t.) administration of the inhibitor produced hyperalgesia in morphine-tolerant mice. In the experiments described here, we sought to characterize further the role of phosphorylation events in supraspinal versus spinal opioid-mediated pain pathways and how such events might be involved in the development of antinociceptive tolerance. Two phosphatase inhibitors were administered centrally to determine whether they affected morphine-induced antinociception in naive or chronically morphine-treated mice. By the i.c.v. route, okadaic acid enhanced morphine-induced antinociception in tolerant mice and produced toxicity by the i.t. route. The calcineurin inhibitor ascomycin had no effect on antinociception following acute or chronic morphine treatment. These results suggest that increased activity of protein phosphatase types 1 and/or 2A in the brain may contribute to the development of morphine tolerance. © 1998 Elsevier Science B.V.

Keywords: Opioid; Antinociception; Tolerance; Protein phosphatase; Protein kinase

1. Introduction

Systemically administered morphine produces antinociception via action at both spinal and supraspinal sites (Barton et al., 1980). Morphine activates descending systems within the brainstem that inhibit dorsal horn nociceptive neurons (Basbaum and Fields, 1984) as well as directly inhibiting spinal cord neurons to prevent transmission to supraspinal centers (Yaksh and Noueihed, 1985). The development and expression of tolerance to morphine-induced antinociception may involve either or both the descending and ascending components of the pain pathway. Recent studies suggest that phosphorylation events may play an integral role in the development and expression of tolerance to the inhibitory effects of opioids. Recently a number of in vivo studies have been conducted using newly available, highly specific protein kinase inhibitors. These studies provide evidence that phosphorylation events are either affected by chronic opioid exposure and/or that these events participate in the neuron's response to chronic opioids. Narita et al. (1994) found that concurrent intracerebroventricular (i.c.v.) infusion of morphine and H-7 [1-(5-isoquinolinesulfonyl)-2-methylpiperazine], a relatively nonspecific serine/threonine kinase inhibitor, dose-dependently blocked the development of tolerance to morphine-induced antinociception. More recently, in our laboratory, we have shown that i.c.v. administration of the selective protein kinase A inhibitor KT5720 (a modification of the compound K-252a isolated from *Nocardiopsis* sp.) reverses tolerance to morphine-induced antinociception, suggesting that elevated activity of protein kinase A in the brain is critical to the expression of morphine tolerance (Bernstein and Welch, 1997). By contrast, intrathecal (i.t.) administration of KT5720 produced hyperalgesia. A more extensive literature exists for studies of acute tolerance development than for chronic tolerance to morphine. For example, Wang et al. (1994) and Bilsky et al. (1996) showed that H-7 reverses acute morphine tolerance. Narita et al. (1995) found that calphostin C, a protein kinase C inhibitor, blocks the development of acute tolerance to i.t. [D-Ala^2, *N*-Me-Phe^4, Gly-ol^5]-enkephalin (DAMGO)-induced antinociception, but KT5720, the protein kinase A inhibitor, has no effect in this paradigm.

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Although the mechanisms for acute tolerance development may differ from those of chronic tolerance development, it appears likely that phosphorylation events contribute in both cases.

Because blockade of protein kinases has been shown to reverse tolerance, we hypothesized that blockade of protein phosphatases might potentiate tolerance by prolonging the phosphorylated state of proteins. Two compounds were tested: okadaic acid, which inhibits phosphatases 1 and 2A, and ascomycin, which inhibits type 2B.

2. Materials and methods

All studies were performed in male ICR mice (28–30 g). Tolerance to morphine was induced by anesthetizing mice briefly with ether and implanting morphine (75 mg) or placebo pellets (National Institute on Drug Abuse) under the skin of the back. Incisions were closed with surgical staples. Use of the morphine pellets alone produced only a 2-fold shift in the ED_{50} for s.c. morphine-induced antinociception. To improve this response, s.c. injections were added to the protocol. Approximately 12 h after surgery, the mice received a subcutaneous (s.c.) injection of morphine (20 mg/kg) or distilled water (vehicle) and then received injections every 12 h for a total of 4 days. This treatment protocol produced a consistent, 4-fold shift in the ED_{50} for morphine antinociception (Bernstein and Welch, 1995).

With pellets still in place, mice were tested for antinociception by the tail-flick procedure (D'Amour and Smith, 1941). Radiant heat applied to the tail automatically cuts off after 10 s to prevent tissue damage and the intensity of the beam is adjusted to produce mean control reaction times of 2–4 s. Baseline tail-flick latencies of all mice used in this study, including those that had been treated chronically with morphine, fell within 2 to 4 s. Antinociception was quantified as the percent maximum possible effect (%MPE) as developed by Harris and Pierson (1964) using the formula $\%MPE = 100 \times [(test - control)/(10 - control)]$. Values were calculated for each mouse using 6 mice per dose, from which the mean effect and S.E.M. were calculated for each dose. %MPE values were compared by unpaired, two-tailed Student's *t*-tests. ED_{50} values and confidence intervals were analyzed using a computerized Macintosh version of the Litchfield and Wilcoxon (1949) method.

On test days, mice received either i.c.v. or intrathecal (i.t.) injections of phosphatase inhibitors or vehicle with concurrent s.c. injection of morphine or vehicle. Morphine sulfate (NIDA) was made up in distilled water. Okadaic acid sodium salt (Calbiochem) was solubilized in distilled water and ascomycin (Calbiochem) was made up in a 1:1:18 ratio of emulphor, dimethyl sulfoxide and distilled water. Okadaic acid solutions were made fresh each day and efforts were made to minimize light exposure.

Intracerebroventricular injections of 5 μl were performed under light ether anesthesia using the method of Pedigo et al. (1975). Intrathecal injections were performed following the protocol of Hylden and Wilcox (1983), in which unanesthetized mice are injected with 5 μl of drug between the L5 or L6 area of the spinal cord with a 30-gauge, 1/2-inch needle.

3. Results

Dose-ranging and timecourse studies were conducted to determine whether the inhibitors administered alone produced behavioral effects, including antinociception. At all doses tested and at varying timepoints ranging from 10 to 60 min, %MPE values following either route of administration were less than 10%. Toxicity was evident at okadaic acid doses of 0.5 nmol or greater by either i.t. or i.c.v. route. No toxicity was noted at any dose, timepoint, or route of administration in the ascomycin studies.

When co-administered with morphine (4 mg/kg, s.c.) in naive mice, okadaic acid (0.05–2.5 nmol/mouse, i.c.v.) had no effect on morphine-induced antinociception. Okadaic acid was injected 10–60 min prior to tail-flick testing, with morphine administered 30 min before testing. Administered by the i.t. route, okadaic acid slightly, but not significantly, enhanced acutely administered morphine in naive mice. A full dose–response curve is shown in Fig. 1. The ED_{50} for s.c. morphine with i.c.v. vehicle (both injections made 30 min prior to testing) is 2.0 mg/kg (C.L. 1.2–3.6); with co-administration of 0.25 nmol okadaic acid, the morphine ED_{50} is 0.9 mg/kg (0.4–2.0).

Fig. 2 depicts the results of experiments in which okadaic acid was administered by the i.c.v. route concurrent with a dose of s.c. morphine that alone will produce less than a 50% effect (4 mg/kg in placebo-treated mice and 10 mg/kg in morphine-tolerant mice). As shown in Fig. 2B, okadaic acid alone (0.25 nmol, i.c.v., 30 min)

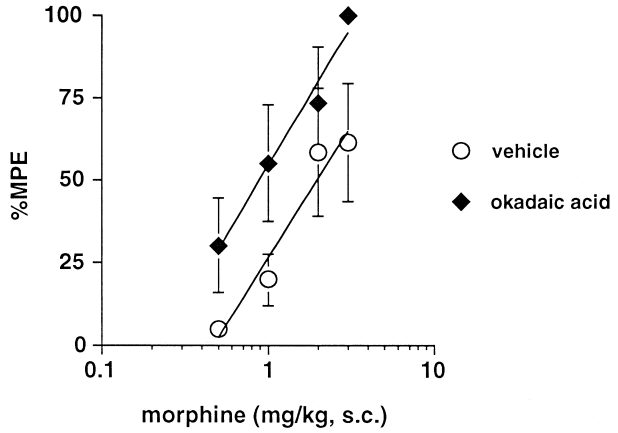

Fig. 1. Dose–response curve in drug-naive mice for s.c. morphine-induced antinociception at 30 min in mice co-treated with i.t. okadaic acid (0.25 nmol/mouse) or vehicle. For each point, $n = 6$ mice.

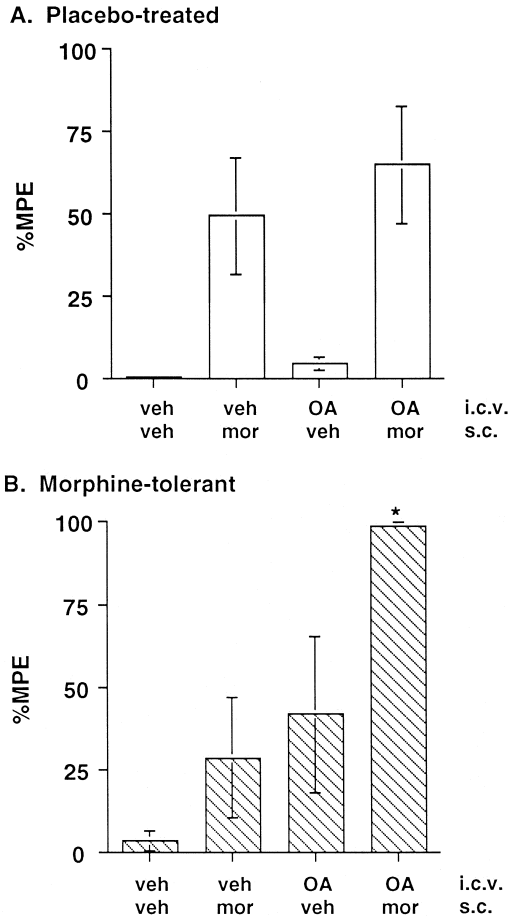

Fig. 2. Effects of i.c.v. okadaic acid (0.25 nmol/mouse) on s.c. morphine-induced antinociception in chronically treated mice. OA = okadaic acid, Mor = morphine, veh = vehicle. Morphine doses used in this experiment were 10 mg/kg for morphine-tolerant mice and 4 mg/kg for placebo controls. For each point, $n = 6$ mice. * OA/mor versus veh/mor and OA/mor versus OA/veh are significantly different from each other, $P < 0.05$.

produced slight but not statistically significant antinociception in morphine-tolerant mice (%MPE = 41.6 ± 23.9, $n = 5$ versus 3.4 ± 3.1, $n = 6$, in vehicle-challenged morphine-tolerant mice). As shown in Fig. 2A, no effect was observed for i.c.v. okadaic acid alone in placebo-treated mice (%MPE = 4.3 ± 1.9, $n = 6$). In morphine-tolerant mice, okadaic acid (0.25 nmol, i.c.v.) co-administered with a challenge dose of morphine (10 mg/kg, s.c.) resulted in significantly higher antinociception than for morphine-tolerant mice receiving morphine without the inhibitor (%MPE = 98.1 ± 1.4, $n = 6$ versus 28.5 ± 18.4, $n = 5$; $p < 0.05$). A similar co-treatment effect was not observed in the placebo-treated animals (Fig. 2A). In addition, in the morphine-tolerant mice, the combined effects of i.c.v. okadaic acid and s.c. morphine was significantly greater than the antinociception produced by okadaic acid alone ($P < 0.05$). By contrast, when injected i.t. in morphine-tolerant but not placebo-treated mice, okadaic acid produced significant toxicity at all doses tested (e.g. seizures and barrel rolling).

Parallel studies were conducted with ascomycin, using doses of drug ranging from 0.05 to 5 nmol. By the i.c.v. route of administration, ascomycin had no effects on either acute morphine antinociception or on the expression of morphine tolerance. Intrathecally administered ascomycin (5 nmol/mouse) slightly blocked morphine-induced antinociception in naive mice, but this effect was not significant at any timepoint tested. For example, with concurrent administration of i.t. ascomycin and s.c. morphine at 30 min prior to testing, the morphine ED_{50} for vehicle-treated mice was 2.8 mg/kg (C.L. 1.3–5.8), while that for ascomycin-treated mice was 4.4 mg/kg (C.L. 2.4–8.0). In chronically treated animals, this nonsignificant trend was also apparent in both control and morphine-tolerant mice. For example, in placebo-treated mice, ascomycin (5 nmol/mouse, i.t.) in combination with morphine (8 mg/kg) produced an %MPE of 63.3 ± 15.9 versus 83.5 ± 11.5 in mice pretreated with vehicle (i.t.); in morphine-tolerant mice, the same doses of ascomycin and morphine produced an %MPE of 9.0 ± 5.8 versus 34.2 ± 15.1 in morphine-tolerant mice pretreated with vehicle ($n = 6$ for all groups).

4. Discussion

The phosphorylation state of a protein is the product of both the rate of phosphorylation by kinases, as well as the rate of dephosphorylation by phosphatases. While relatively less is known about phosphatases than kinases, it is evident that intracellular regulation of phosphatase activity is extremely complex, involving a combination of targeting and regulatory subunits as well as endogenous inhibitors (Cohen and Cohen, 1989). Because the intrinsic catalytic activity and intracellular concentrations of most kinases and phosphatases are approximately the same (Cohen, 1992), inhibition of phosphatase activity will tend to promote the phosphorylated state of substrate proteins. If enhanced kinase activity contributes to the expression of opioid tolerance, then inhibited phosphatase activity might further enhance the degree of tolerance.

Okadaic acid is a shellfish toxin that potently inhibits serine/threonine-specific protein phosphatases and can penetrate cells easily. Okadaic acid inhibits phosphatases 1 (IC_{50} = 10–15 nM) and 2A ($IC_{50} < 1$ nM) as well as types 4 and 5, with maximum blockade of all these phosphatases achieved at 1 μM (Cohen and Cohen, 1989; Haystead et al., 1989). Ascomycin is a compound related to the immunosuppressants cyclosporin A and FK-506, which potently inhibit calcineurin, serine/threonine-specific phosphatase type 2B ($IC_{50} < 10$ nM) (Liu et al., 1991; Shafiee et al., 1993).

Our hypothesis for these studies was that protein phosphatase inhibition would potentiate the expression of toler-

ance, since it has been shown in our lab and others that inhibition of protein kinases reverses the expression of tolerance to morphine antinociception (Bernstein and Welch, 1997; Narita et al., 1994). Therefore we were surprised to find a slight but significant effect of i.c.v. okadaic acid in the opposite direction of that predicted. Okadaic acid administered alone produced some antinociception in morphine-tolerant mice, although this was not statistically different from that of the vehicle control group (Fig. 2). It would appear that following okadaic acid administration, these mice became resensitized to the morphine already in their system from the 4-day treatment, which suggests that phosphatase inhibition returned one of more of the critical substrate proteins to its basal phosphorylation state. It would be useful to co-administer okadaic acid chronically with morphine to determine if it might enhance or block tolerance development.

In our previous study employing kinase inhibitors administered either supraspinally or spinally, we found that blockade of phosphorylation in the spinal cord produced hyperalgesia in the morphine-tolerant mice only (Bernstein and Welch, 1997). Here we find that manipulation of spinal phosphatase activity produces toxicity. Previously we suggested that these results may stem from differential μ-opioid receptor (μ_1 versus μ_2) subtype activation in the brain as compared with spinal sites. It has also been shown that chronic i.t. administration of morphine produces hyperalgesia (Mao et al., 1994), which may become apparent in the case of systemic morphine administration when it is further enhanced by inhibition of phosphorylation activity. It is also possible that chronic mu opioid exposure leads to decreased activity of both kinases and phosphatases in the spinal cord, such that when either are exogenously inhibited, the sudden imbalance produces toxicity.

Central injection of inhibitor compounds provides limited information, primarily because it is unclear which proteins are being affected and what brain regions are involved. The advantage of this technique is that the effect of inhibition is shown by a behavioral measure, which permits evaluation of antinociceptive tolerance. It appears that the balance between kinases and phosphatases can be disrupted by blocking one or the other, showing that either may be altered with tolerance development. However, because phosphatases can affect the activity of kinases (Cohen, 1992), blockade of phosphatase activity may not only impede dephosphorylation but actually enhance phosphorylation. Our data from this and previous experiments show that both kinase and phosphatase activity are changed with tolerance. Therefore, it is unlikely that a uniform shift occurs throughout the neuron of all proteins toward a phosphorylated state, but rather a mixed response takes place. This conclusion is supported by in vitro studies, in which some researchers report increases in the number of phosphoproteins following chronic opioid treatment (e.g. Terwilliger et al., 1991) while others report decreased phosphoprotein levels (e.g. Ehrlich et al., 1978).

Another caveat of central administration of inhibitors is the issue of dosage and resulting drug concentration at the site of action. The doses of drug used in this study were fairly high relative to their IC50 values. Assuming the mice have a cerebrospinal fluid volume of 100 μl, the drug concentration is approximately 1–10 mM at the highest doses used in this study. However, we cannot be sure that all of the administered compound entered the neurons or was freely distributed. At least in the case of okadaic acid, the production of neurotoxicity is evidence that the inhibitor penetrated the neuron, although the specificity of its activity is uncertain. The toxicity of okadaic acid limits the conclusions that can be drawn from this study.

While changes occur in kinase activity in the brains of morphine-tolerant animals, it also appears that compensatory changes occur in phosphatase activity with tolerance development. Such changes appear to take place supraspinally, and perhaps spinally as well. As shown in our previous study using kinase inhibitors (Bernstein and Welch, 1997), during opioid tolerance the spinal cord becomes extremely sensitive to drugs that affect phosphorylation, which is again apparent with phosphatase inhibition. Further studies are needed, particularly in vitro, to clarify the role played by protein phosphatases in opioid tolerance.

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