Acute Tolerance To Spinally Administered Morphine Compares Mechanistically with Chronically Induced Morphine Tolerance

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ABSTRACT

The mechanistic similarity between acutely and chronically induced morphine tolerance has been previously proposed but remains largely unexplored. Our experiments examined the modulation of acutely induced tolerance to spinally administered morphine by agonists that affect the N-methyl-D-aspartate receptor and nitric oxide synthase systems. Antinociception was detected via the hot water (52.5°C) tail flick test in mice. Intrathecal pretreatment with morphine (40 nmol) produced a 9.6-fold rightward shift in the morphine dose-response curve. This shift confirmed the induction of acute spinal morphine tolerance. Intrathecal copretreatment with the receptor antagonists (competitive and noncompetitive, respectively) dizolcipine (MK801, 3 nmol) or LY235959 (4 pmol) and morphine [40 nmol, intrathecally (i.t.)] attenuated acute tolerance to morphine measured 8 hr later. A 60-min pretreatment of 7-nitroindazole (6 nmol, i.t.), a selective neuronal NOS inhibitor, followed by administration of morphine (40 nmol, i.t.) blocked the induction of morphine tolerance. Intrathecal copretreatment with morphine (40 nmol, i.t.) and agmatine (4 nmol, i.t.), an imidazoline, receptor agonist and putative nitric oxide synthase inhibitor, almost completely abolished acute spinal morphine tolerance. The results of these experiments agree with previous reports using models of chronically induced morphine tolerance. This evidence supports the proposal that the mechanisms responsible for acute morphine tolerance parallel those underlying chronic morphine tolerance. This study attests to the powerful predictive value of acute induction as a model for morphine tolerance.

The mechanism by which morphine tolerance develops has yet to be definitively described and continues to be intensely investigated. Comparison of results across studies is difficult due to profound differences in study design. These include, for example, route of administration (e.g., spinal vs. systemic), method of induction (e.g., acute vs. chronic), test subject species (e.g., mouse vs. rat), measure of opioid action (e.g., tail flick vs. hot plate), strain differences within species (Rady et al., 1991), and source differences within strains (Clark and Proudfit, 1992).

The induction of morphine tolerance appears to have two phases: an acute component and a chronic state (Rosenfeld and Burks, 1977). Acute tolerance develops within hours after a single bolus dose of opioid agonist (Yano and Take-mori, 1977) and persists at least 48 hr (Huidobro-Toro et al., 1978). The majority of morphine tolerance studies investigate chronic tolerance, which typically requires administration of agonist for 3 to 7 days. However, current chronic induction methods carry notable drawbacks that may limit interpretation. In many studies opioid agonists have been administered by repeated injection either systemically or directly into the central nervous system over a period of 3 to 7 days. Under these conditions, maintaining a constant plasma or cerebrospinal fluid drug concentration can be difficult or impossible to achieve; the animals may enter withdrawal states repeatedly when agonist levels fall between injections (Sparber et al., 1979, 1978; Stevens, 1994). Repeated injections have also been associated with a learning or associative component that may present difficulties in the interpretation of the results (Ben-Eliyahu et al., 1992; Siegel, 1988).

A second commonly used method of chronic morphine tolerance induction requires morphine pellet implantation. This technique may be accompanied by systemic illness and stress (Sparber et al., 1979); furthermore differences in pellet hardness and absorbability (Meyer and Sparber, 1976), pellet composition and drug release kinetics (Blasig et al., 1973) and fibrous encapsulation (Stevens, 1994) may confound comparisons between results from different laboratories. A third method, chronic intrathecal infusion, may minimize

ABBREVIATIONS: ANOVA, analysis of variance; CREB, cAMP-responsive element-binding protein; I1, imidazoline; i.t., intrathecally; LNN, L-N(3)-nitro-L-arginine; L-NAME, L-N(3)-nitro-L-arginine methyl ester; LY, LY235959; MK801, dizolcipine; % MPE, percent maximum possible effect; NMMA, N(3)-monomethyl-L-arginine; NO2Arg, N(3)-nitro-L-arginine; NO, nitric oxide; NOS, nitric oxide synthase; 7-NI, 7-nitroindazole; NMDA, N-methyl-D-aspartate; pmol, picomoles; PKC, protein kinase C.
chronic side effects such as systemic illness, motor dysfunction and stress, but can be confounded by displaced (Wiesenfeld and Gustafsson, 1982) or encapsulated (Sabbe et al., 1988) catheters. Encapsulation may result in diffusion barriers preventing the agonist reaching its intended spinal site of action (Coombs et al., 1985; Samuelsson et al., 1987). Additionally, chronic i.t. catheterization may induce neurochemical changes in the central nervous system (Millan et al., 1989; Rovati et al., 1988).

Opioid-releasing cell implantation models (Wu et al., 1994) and adrenal medullary tissue implant models (Wang and Sagen, 1994) have recently been developed to address some of these problems but are accompanied by uncertainty as to which released agents (β-endorphin, nicotine, ACTH or growth factors) may induce or alter the development of tolerance. Finally, some pharmacological agents useful in isolating the mechanisms of tolerance induction may be found to induce toxicity, contraindicating their use in chronic tolerance studies. For example, the protein kinase inhibitors H7 and H8 are reported to be toxic in chronic dosing schedules (Bilsky et al., 1996a). A second example of toxicity-limited tools includes protein synthesis inhibitors, which induce metabolic disturbances when administered chronically (Young et al., 1963). Conditions such as these are likely to confound the interpretation of results and lead us to consider alternatives for the study of tolerance.

Comparatively fewer studies have employed acute or single-dose tolerance (Cox et al., 1968; Huidobro-Toro and Way, 1978; Kissin et al., 1991ab; Narita et al., 1995; Nielsen and Sparber, 1985; Song and Takemori, 1992; Vaught et al., 1981). Acute tolerance mitigates many of the limitations present in chronic induction schedules. Potency changes, revealed by significant rightward shifts of the probe morphine dose-response curve, persisting for as long as 48 hr (Huidobro-Toro and Way, 1978), demonstrate the robust development of acute tolerance. Full agonist efficacy remains attainable at higher doses after toleragen (tolerance-inducing agent, i.e., morphine) administration, distinguishing acute tolerance from tachyphylaxis which involves reduced efficacy as well. The method of induction remains simple whether the route of administration is systemic or i.t. Subject animals do not present symptoms of illness or disability. The investigator maintains substantially improved control of drug dose and the time course characteristics are more specifically and accurately observed.

Studies selectively examining the spinal mechanisms of opioid action represent a small subset of the substantial tolerance literature. However, there exists a critical clinical need to understand fully the spinal mechanism of morphine tolerance. A variety of clinical techniques (epidural and spinal catheterization; implantable pump) use direct spinal administration of opiates in an attempt to treat intractable cancer pain (Behar et al., 1979; Coombs et al., 1985; Cousins et al., 1979; Onofrio et al., 1981; Samuelsson et al., 1995; Wang et al., 1979). Clinical practice, therefore, underscores the need for continued and expanded experimental exploration of the mechanisms governing spinal opioid tolerance. Techniques such as spinal catheterization of the subarachnoid space (Yakh and Rudy, 1976) and intrathecal injection (Hylden and Wilcox, 1980) permit the isolation of the spinal site of opioid action in conscious animals. This isolation provides the means to determine the sites of opioid agonist action within the central nervous system: that is, to differentiate how opioids act in spinal cord vs. supraspinal sites and which descending tracts may modulate such action. Subsequent studies using these techniques clearly implicated spinal cord as a critical site in the development of morphine tolerance (DeLander et al., 1984; Delander and Takemori, 1983; Yaksh et al., 1977).

Many behavioral studies of spinal morphine tolerance use the rat as a model; one rationale for this choice has been that the rat’s larger size facilitates implantation of infusion catheters. However, chronic catheter studies remain impractical in mice. Species selection becomes an important issue in view of the recent introduction of mutant, gene-targeted mouse lines that present expanded opportunities for the study of mechanism in vivo. This advance calls for the establishment of a reliable murine model for the investigation of mechanisms of spinal morphine tolerance; the acute tolerance paradigm serves this need effectively. Elucidation of the similarities and differences between acute and chronic spinal morphine tolerance may advance our understanding of the fundamental mechanisms underlying morphine tolerance (Mucha and Kalant, 1980). Toward this end, we applied a mouse model of acute induction of tolerance to spinally administered morphine by intrathecal injection.

To test the generalizability of acute morphine tolerance to chronic morphine tolerance, we elected to test a series of agents known to modulate chronic morphine tolerance. The prevention of the development of chronic morphine tolerance and/or dependence by NMDA receptor antagonists (Ben-Eliyahu et al., 1992; Elliott et al., 1994a, b; Marek et al., 1991; Tiseo et al., 1994; Tiseo and Inturissi, 1993; Trujillo and Akil, 1991) and NOS inhibitors (Elliott et al., 1994b; Kolesnikov et al., 1992, 1993; Majeed et al., 1994; Vaupel et al., 1995a) is well-established in the literature. Recent evidence demonstrates that NMDA receptor antagonist-mediated modulation of opioid tolerance may be selective to morphine induction and not observed with induction by other opioid ligands (Bilsky et al., 1996c). Therefore, these modulators are well-suited for investigating the parallels between acute morphine tolerance and chronic morphine tolerance.

The present experiments demonstrate the ability of NMDA receptor antagonists and NOS inhibitors to attenuate or abolish the induction of acute tolerance to spinally administered morphine. These experiments consistently reveal results similar to those reported in chronic systemic induction models. These observations suggest that information about mechanism obtained through acute morphine tolerance studies will correlate closely with comparable information from studies of chronic morphine tolerance.

Materials and Methods

Animals. All experimental subjects were 20 to 25 g male ICR mice (Harlan Sprague Dawley, Indianapolis, IN). These experiments were approved by the Institutional Animal Care and Use Committee. All animals were housed in groups of 10 in a temperature- and humidity-controlled environment for 4 to 5 days before experimentation. All animals were maintained on a 12-hr light/dark cycle and had free access to food and water. Each animal was used only once.

Chemicals. Morphine sulfate was a gift of Dr. R. P. Elde (University of MN), dizocilpine (a noncompetitive NMDA receptor antagonist, MK801) was a gift of Merck Chemical Co. (Rahway, NJ); LY235959 (a competitive NMDA antagonist) was a gift of Eli Lilly.
Antinociceptive testing. Nonciceptive responsiveness was determined using the warm water (52.5°C) immersion tail flick test. The latency to the first rapid tail flick represented the behavioral endpoint (Janssen et al., 1963). Baseline measurements of tail-flick latencies were collected on all subjects (for a sample of n = 817, x = 4.0, S.D. = 1.3). Mice that failed to respond within 5 sec to baseline tests were excluded from analysis (18%). The % MPE was determined according to the following formula: % MPE = (postdrug latency-predrug latency)/cutoff-predrug latency) × 100%. To avoid tissue injury, a maximum score of 100% was assigned to those animals not responding within 12 sec. Drugs were injected i.t. by direct lumbar puncture (Hyliden and Wilcox, 1980).

Acute tolerance induction. Mice were made acutely tolerant to morphine by a single intrathecal injection of morphine, in most cases at a dose of 40 nmol except as noted. All injections were administered between 06:00 and 09:00 hr. Approximately 8 hr after the injection, tail-flick latencies were collected on all subjects to determine that the tail flick latencies had returned to baseline levels (for a sample of n = 251, x = 3.1, S.D. = 0.9). Those animals that failed to respond within 5 sec to the tail flick test were excluded from analysis (4%). Subjects were then challenged with varying doses of morphine (0.2, 0.6, 2, 8, 15, 20 nmol, i.t.). The tail flick test was performed 10 min after this probe morphine injection. Dose-response curves were generated and ED<sub>50</sub> values and confidence limits were calculated according to the method of Tallarida and Murray (1987). Groups of 7 to 10 animals were used for each dose and/or each pretreatment.

NMDA receptor antagonist modulation of acute spinal morphine tolerance. Dizocilpine (MK801, 3 nmol, i.t.) and LY235959 (4 pmol, i.t.) were each coadministered with morphine (0.2, 0.6, 2, 8 nmol, i.t.) to test for possible modulatory effects on the acute antinociceptive effects of morphine. Dose-response curves for the antinociceptive effects of these coadministered agents were generated. To test for the NMDA receptor antagonists’ modulatory effect on the induction of acute tolerance to spinally administered morphine, mice were either copretreated with morphine (40 nmol, i.t.) together with the NMDA receptor antagonist dizocilpine (MK801, 3 nmol, i.t.) or LY235959 (4 pmol, i.t.). Approximately 8 hr later, animals were challenged with varying doses of morphine (MK801: 0.2, 0.6, 2, 8 nmol morphine, i.t.; LY235959: 0.8, 2, 5, 8 nmol morphine, i.t.). The tail flick test was performed before and 10 min after this probe morphine injection, and morphine probe antinociceptive dose-response curves generated.

NOS inhibitor 7-NI modulation of acute spinal morphine tolerance. Vehicle (peanut oil) and 7-NI (6 nmol, i.t.) dissolved in vehicle were each coadministered with morphine (0.2, 0.6, 2, 8 nmol, i.t.) to test for possible modulatory effects on the acute antinociceptive effects of morphine in the tail flick test. Dose-response curves to the antinociceptive effects of these coadministered agents were generated. To test for the modulatory effect of 7-NI on the induction of acute tolerance to spinally administered morphine, mice were copretreated with morphine (40 nmol, i.t.) together with 7-NI (6 nmol, i.t.), a dose sufficiently high to block the NMDA-induced hyperalgesia (data not shown) observed previously (Kitto et al., 1992). Approximately 8 hr later, animals were challenged with varying doses of morphine (0.2, 0.6, 2, 8 nmol morphine, i.t.). The tail flick test was performed before and 10 min after this probe morphine injection and morphine probe antinociceptive dose-response curves were generated.

Agmatine modulation of acute spinal morphine tolerance. Agmatine (0.2, 0.6, 2, 8, 20, 30, 100 nmol, i.t.) was tested for possible antinociceptive effects in the tail flick test. Agmatine (4 nmol, i.t.) coadministered with morphine (0.2, 0.6, 2, 8 nmol, i.t.) was tested for possible modulatory effects on the acute antinociceptive effects of morphine in the tail flick test. Mice were copretreated with morphine (40 nmol, i.t.) together with agmatine (4 nmol, i.t.) and morphine probe antinociceptive dose-response curves were generated. This experiment was replicated twice, once unblinded and once blinded; both experiments yielded similar results. As a control for agmatine’s activity at the putative imidazoline receptor, mice were copretreated with morphine (40 nmol, i.t.) together with moxonidine (1 nmol, i.t.) and a morphine probe antinociceptive dose-response curve was generated.

Modulatory agent’s effect on the induction of tolerance to morphine. To test whether each modulatory agent could induce “tolerance” in the absence of morphine toleragen (40 nmol, i.t.), single groups of mice (n = 9–10) received i.t. injections with each agent (MK801: 3 nmol; LY235959: 4 pmol; 7-NI: 6 nmol; agmatine: 4 nmol; moxonidine: 1 nmol; peanut oil, 5 μl; saline; or morphine, 40 nmol). Approximately 8 hr later, animals were challenged with a single probe dose of morphine (8 nmol, i.t.). The tail flick test was performed before and 10 min after this probe morphine injection. The means of the maximum possible effect (% MPE) values of the groups that received the modulatory agent or vehicle were tested for significance using ANOVA and statistical differences between the groups were further analyzed with Dunnett’s test for multiple comparisons to a control (saline group).

Statistical analysis. Data describing antinociception are expressed as means of percent maximal possible effect (% MPE) with S.E.M. For experiments testing responses to a single probe dose, statistical significance was evaluated using Student’s t test (significance set at P < .05). Potency changes are presented as ED<sub>50</sub> value dose ratios between the ED<sub>50</sub> values of different dose-response curves. However, statistical comparisons of potencies are based on the confidence limits of the ED<sub>50</sub> values. The ED<sub>50</sub> values and confidence limits were calculated according to the method of Tallarida and Murray (1987). Groups of 7 to 10 animals were used for each dose and/or each pretreatment.

Results

Confirmation of the induction of acute tolerance to i.t.-administered morphine. To characterize the dose-response relationships present in acute spinal tolerance, we initially determined dose-response curves for the antinociceptive effects of morphine in the tail flick test in naïve, saline-pretreated and morphine-pretreated (10 and 40 nmol, i.t.) mice. Morphine dose-response curves did not differ between naïve or saline-pretreated mice (fig. 1A). Data from two naïve and one saline-pretreated dose-response curves were pooled to generate a nontolerant dose-response curve (fig. 1B, ED<sub>50</sub> 1.2 nmol, 0.9–1.7). Dose points included in the pooled curves represent groups with more than 10 subjects. Morphine pretreatment increased the ED<sub>50</sub> value in a dose-dependent manner (fig. 1A). Pretreatment with 10 nmol morphine (i.t.) produced a 3-fold rightward shift in the morphine dose-response curve (ED<sub>50</sub> 3.7, 2.0–6.6) (fig. 1A). This ED<sub>50</sub> value was calculated from the doses included in the monotonic portion of the dose-response curve. Data from three morphine-pretreated (40 nmol) dose-response curves (fig. 1A) were pooled and are represented in figure 1B. Pretreatment with 40 nmol morphine produced a 9.6-fold rightward shift in the morphine dose-response curve (ED<sub>50</sub> 12 nmol, 8.4–16). This dramatic rightward shift confirms the induction of morphine tolerance in this acute model. Acute spinal morphine tolerance demonstrates reliable replicability in this model (fig. 1A).
Attenuation of acute spinal tolerance by NMDA receptor antagonists. The attenuation of chronically induced opioid tolerance by NMDA receptor antagonists has been well established (Ben-Eliyahu et al., 1992; Elliott et al., 1994b; Marek et al., 1991; Tiseo et al., 1994; Tiseo and Inturissi, 1993; Trujillo and Akil, 1991). We tested the modulatory effects of two NMDA receptor antagonists on acute spinal morphine tolerance. Morphine (0.2, 0.6, 2, 8 nmol, i.t.) administered to mice copretreated with morphine (40 nmol, i.t.) together with dizocilpine (MK801, 3 nmol) produced an antinociceptive dose-response curve with an ED$_{50}$ value of 2.1 nmol (1.0–4.9) (fig. 2B). This ED$_{50}$ value differs substantially from that of the morphine-pretreated dose-response curve (ED$_{50}$: 12 nmol (8.4–16)). This demonstrates that dizocilpine effectively attenuates the development of acutely induced tolerance to spinally administered morphine. Morphine (0.8, 2, 5, 8 nmol, i.t.) administered to mice copretreated with morphine (40 nmol, i.t.) together with LY235959 (4 pmol) produced a 4.6-fold rightward shift in the antinociceptive dose-response curve with an ED$_{50}$ value of 5.5 nmol (3.1–9.5) (fig. 3B) which differs significantly from that of morphine pretreatment group (ED$_{50}$: 12 nmol, 8.4–16). This difference demonstrates that LY235959 effectively attenuates the development of acutely induced tolerance to spinally administered morphine. Coadministration of morphine (0.2, 0.6, 2, 8 nmol, i.t.) in the presence of dizocilpine (MK801, 3 nmol, i.t.) or LY235959 (4 pmol, i.t.) in naive animals produced no potency shift compared to morphine administered alone to naive/saline-pretreated animals (figs. 2A and 3A). This demonstrates that these antagonists do not modulate acute mor-

Fig. 1. Confirmation of the induction of acute spinal morphine tolerance. A. Dose-response curves of the spinal antinociceptive effect of morphine on naive (closed circles, closed squares), saline-pretreated (closed triangles) and morphine-pretreated at 10 nmol (open diamonds) and 40 nmol (open circles, open squares, open triangles). B. Dose-response curves from pooled data of the three nontolerant curves (open squares) and the three morphine tolerant (40 nmol pretreatment, closed triangles) curves presented in A. Dose points presented include only those with an n > 10 animals. The pooled dose-response curves are presented in subsequent figures as benchmarks of tolerance and naive/saline-pretreated morphine dose-response curves to facilitate comparison against the modulatory agents. The pooled dose-response curve for morphine-pretreated animals reveals an ED$_{50}$ value of 1.2 nmol (0.9–1.7). The pooled dose-response curve for morphine-pretreated animals reveals an ED$_{50}$ value of 12 nmol (8.4–16). These results confirm the induction of acute tolerance to i.t. administered morphine.

Fig. 2. Noncompetitive NMDA receptor antagonist dizocilpine (MK801) copretreatment with morphine attenuates the development of acute spinal morphine tolerance. Dose-response curves for morphine-induced antinociception from pooled naive/saline-pretreated (open squares) and morphine-pretreated (closed triangles) are represented in A and B as described in 1B. A. MK801 (3 nmol, i.t.) coadministration with morphine (0.2, 0.6, 2, 8 nmol, i.t.) produced a morphine dose-response curve with an ED$_{50}$ value of 0.7 nmol (0.2–2.5), (closed circles). Coadministration of MK801 with morphine does not modulate acutely tested morphine antinociception. B. MK801 (3 nmol, i.t.) coadministration with morphine (40 nmol, i.t.) as a pretreatment subsequently tested with probe morphine (0.2, 0.6, 2, 8 nmol, i.t.) produced a morphine dose-response curve with an ED$_{50}$ value of 2.1 nmol (1.0–4.9) (closed circles). Coadministration of MK801 with morphine toleragen effectively prevents the development of acute tolerance to intrathecally administered morphine.
phine antinociception. An 8-hr pretreatment with either dizocilpine (3 nmol, i.t.) or LY235959 (4 pmol, i.t.) did not effect a difference in the efficacy of morphine (8 nmol, i.t.) when compared to saline-pretreated control group (p < .05 in both cases, data not shown).

**Prevention of acute spinal tolerance by 7-NI, an NO synthase inhibitor.** The attenuation of chronically-induced morphine tolerance by NOS inhibitors has been demonstrated previously (Bhargava, 1995; Elliott et al., 1994; Kolesnikov et al., 1993; Kolesnikov et al., 1992; Majeed et al., 1994). We tested 7-NI for its ability to block acutely induced tolerance to spinally administered morphine. Morphine (0.2, 0.6, 2, 8 nmol, i.t.) administered to mice pretreated with 7-NI (6 nmol, i.t.) 1 hr before the tolerance-inducing pretreatment with morphine (40 nmol, i.t.) produced an antinociceptive dose-response curve with an ED50 value of 0.3 nmol (0.1–0.8) (fig. 4B). This ED50 value represents a 4-fold leftward shift in the morphine dose-response curve from that of naive/saline-pretreatment group (ED50 1.2 nmol (0.9–1.7)). This demonstrates that 7-NI pretreatment effectively blocks the development of acutely induced tolerance to spinally administered morphine. Administration of morphine (0.2, 0.6, 2, 8 nmol, i.t.) after a 1-hr pretreatment with either vehicle or 7-NI dissolved in vehicle in naive animals produced no potency shift compared to morphine administered alone to naive/saline-pretreated animals (fig. 4A). This result demonstrates that this NOS inhibitor does not modulate acute morphine antinociception. An 8-hr pretreatment with vehicle (peanut oil) or 7-NI (6 nmol, i.t.) dissolved in vehicle did not effect a difference in the efficacy of morphine (8 nmol, i.t.) when compared to saline-pretreated control group (P < .05, data not shown).

**Blockade of acute spinal tolerance by agmatine.** Kolesnikov and colleagues (1996) reported that systemically administered agmatine prevented the development of μ opioid (morphine, s.c) and δ opioid (DPDPE, i.t.) chronically induced tolerance. We tested agmatine for its ability to block acutely induced tolerance to intrathecal morphine. First, we tested agmatine for potential antinociceptive effects. We also tested agmatine for a possible modulatory effect on acute morphine antinociception. Agmatine at varying doses (2, 4, 6, 8, 20, 100 nmol, i.t.) produced no antinociceptive effect in the tail flick test (data not shown). Agmatine (4 nmol, i.t.) did not modulate acute effects of morphine antinociception (0.2, 0.6, 2, 8 nmol, i.t.) on tail flick latency (ED50 1.7 nmol, 1.0–2.9) (fig. 5A). At a higher dose of agmatine (100 nmol, i.t.), antinociception induced by a single dose of morphine (8 nmol, i.t.) was not different between those animals coadministered morphine with agmatine (100 nmol, i.t.) and those administered morphine alone (data not shown). An 8-hr pretreatment with agmatine (4 nmol, i.t.) did not effect a difference in the efficacy of morphine (8 nmol, i.t.) when compared to saline-pretreated control group (P < .05, data not shown). Agmatine (4 nmol, i.t.) administered as a copretreatment with morphine toleragen (40 nmol, i.t.) attenuated the development of morphine tolerance (fig. 5B). The probe morphine dose-response curve (0.2, 0.6, 2, 8 nmol, i.t.) was shifted 2.1-fold to the right (ED50 2.6 nmol, 1.4–4.6) relative to that of saline-pretreated subjects. This ED50 value significantly differs from that of morphine pretreatment group (ED50 12 nmol, 8.4–16) and demonstrates that agmatine robustly attenuates acutely induced tolerance to spinally administered morphine. Moxonidine, a putative imidazoline1 receptor-selective agonist with known central nervous system activity (Fairbanks and Wilcox, 1996; Haxhiu et al., 1994) was tested in this model to control for agmatine’s potential activity at the imidazoline receptor. Moxonidine (1 nmol, i.t.) administered as a copretreatment with morphine toleragen (40 nmol, i.t.) did not modulate the development of morphine tolerance (fig. 5C). Probe administration of morphine (0.2, 0.6, 2, 8, 15, 20 nmol, i.t.) in animals copretreated with moxonidine (1 nmol, i.t.) and morphine (40 nmol, i.t.) produced an ED50 of 19 nmol (8.1–45) comparable to that of subjects pretreated with morphine (40 nmol, i.t.) only (ED50 12 nmol, 8.4–16). An 8-hr pretreatment with moxonidine (1 nmol, i.t.) did not show a difference in the efficacy of morphine (8 nmol, i.t.)
when compared to saline-pretreated control (P < .05, data not shown). These results suggest that the ability of agmatine to block the development of morphine tolerance is not mediated through imidazoline receptor activation.

Discussion

Tolerance may be defined as a decrease in agonist effect over time and/or a significant rightward shift in the agonist dose-response curve (Stevens, 1996). Acute tolerance appears within hours of agonist administration and lasts at least 2 days (Huidobro-Toro and Way, 1978). In our model, a single i.t. bolus dose of morphine produced a statistically significant and dose-related rightward shift of the morphine dose-response curve (fig. 1). This result confirms the induction of acute tolerance by this route of administration, which has been previously implemented in other studies (Narita et al., 1995). Our study explored the modulatory effects on acute tolerance to intrathecally administered morphine of four agents implicated in the modulation of the NMDA receptor/NOS cascade. These experiments yielded results comparable to those reported in chronic induction models. Taken collectively, these observations strongly support the hypothesis that acute tolerance is mechanistically comparable to chronic tolerance.

NMDA receptor antagonist modulation of acute spinal morphine tolerance. Trujillo and Akil (1991) demonstrated that rats made tolerant to morphine through repeated injections (b.i.d., s.c.) for up to 9 days became tolerant to the antinociceptive effects of morphine in the tail flick assay. However chronic pretreatment by repeated injection (i.p.) with MK801, a noncompetitive NMDA receptor antagonist, dose-dependently attenuated morphine tolerance. Tiseo and Inturissi (1993) extended this investigation to LY274614, a competitive NMDA receptor antagonist that does not induce the PCP-like side effects seen with MK801 (Rasmussen et al., 1991). In their study, pretreatment with LY275414 prevented the development of morphine tolerance and reversed preexisting morphine tolerance. LY275414 (24 mg/kg/24 hr) was administered by continuous infusion (s.c.) to rats, morphine antinociception was measured by the hot plate test, and morphine tolerance was induced by repeated injection (10 mg/kg b.i.d., s.c.) for 7 days. Animals treated with LY275414 showed a full antinociceptive response to morphine (10 mg/kg) on days 3 and 6 although tolerance to morphine (10 mg/kg, s.c.) was already evident by the third day in control subjects. Elliott and colleagues (1994b) extended this investigation to mice; MK801 (i.p.) and LY275414 (i.p., s.c.) attenuated the chronic induction of morphine tolerance (repeated injection once daily, s.c.) as determined by the tail-flick method on day 5. Ben-Eliyahu and colleagues (1992) tested the ability of MK801 to attenuate tolerance to morphine induced by a single injection of a sustained release preparation (s.c.) in rats. By this method of induction, MK801 attenuated the development of tolerance to probe morphine by 24 hr. This blockade was significantly measurable as long as 12 days postinjection. The present experiments demonstrate that blockade of the NMDA receptor at the PCP site by MK801 and the glutamate recognition site by LY235959 re-
results in the attenuation of the development of acutely induced tolerance to spinally administered morphine in mice (figs. 2B and 3B). These findings concur with and extend those observations reported in the chronic studies described above.

Abatement of acute spinal tolerance by NOS modulators. The attenuation of chronically induced morphine effects by NOS inhibitors has been well described. Systemic administration of NO2Arg prevents morphine tolerance (Elliot et al., 1994b; Kolesnikov et al., 1992, 1993; Majeed et al., 1994) and signs of morphine dependence (Kolesnikov et al., 1993; Majeed et al., 1994) in mice systemically administered morphine over 3 to 5 days to several weeks. NO2Arg also reverses preexisting morphine tolerance and dependence (Kolesnikov et al., 1993). Two additional NOS inhibitors, L-NAME (Majeed et al., 1994) as well as NMMA (Bhargava, 1995), have also proven to be effective in preventing the development of tolerance and signs of dependence to morphine administered systemically over 3 to 5 days. 7-NI is a relatively novel inhibitor of NOS that is proposed to be advantageous over other NOS inhibitors for its neuronal NOS selectivity and lack of cardiovascular side effects (Moore et al., 1993). Vaupel and colleagues (1995a) demonstrated that 7-NI effectively attenuated withdrawal behaviors (weight loss, diarrhea, wet dog shakes and grooming) in rats after morphine pellet implantation lasting 3 days. Our study presents the first report that 7-NI robustly attenuates acutely induced tolerance to spinally administered morphine (fig. 4A and B). This finding corroborates reports from the chronic morphine tolerance studies using L-NNA, NO2Arg and L-NAME as tolerance attenuators and complements the chronic dependence evidence that 7-NI diminishes the effects of morphine withdrawal. 7-NI may prove suitable for clinical modulation of opioid withdrawal or tolerance due to its neuronal selectivity and apparent lack of hypertensive effects (Moore et al., 1993; Vaupel et al., 1995b).

Kolesnikov and colleagues (1996) reported that systemically administered agmatine prevented the development of chronically induced μ opioid (morphine, s.c.), and δ opioid (DPDPE, i.t.) tolerance. Agmatine also potentiated morphine antinociception in naive mice producing a 5-fold leftward shift in the morphine ED50 value. Interestingly, our experiments reveal that i.t. administered agmatine does not appear to affect acute morphine (i.t.) antinociception in naive mice (fig. 5A) but clearly prevents the development of acutely induced spinal tolerance (fig. 5B). This suggests that agmatine may modulate opioid tolerance directly at a spinal site, rather than through a descending pathway. The specific mechanism by which agmatine exerts this effect remains to be defined. Agmatine has been implicated in the inhibition of NOS (Auguet et al., 1995; Galea et al., 1996), and therefore NOS modulation would appear to be a likely mechanism for agmatine’s action. However, agmatine has also been shown to block the NMDA receptor (Yang and Reis, 1996) in rat hippocampal neurons. Identification of the specific pathway by which agmatine exerts this effect may lead to novel directions in clinical coadministration of tolerance-modifying agents with opioid agonists. Current coadministration schedules that capitalize on receptor blockade by the NMDA receptor antagonists may be limited by toxic side effects (Vau-
pel et al., 1995b). As an endogenous ligand, agmatine may not present those disadvantages. Consistent with this hypothesis, in our study, mice intrathecally injected with agmatine at doses up to 100 nmol did not show overt signs of toxicity such as hindlimb paralysis, hyperlocomotion or sedation. Regardless of the mechanistic source of its action, our study demonstrates that intrathecally administered agmatine robustly attenuates the induction of acute morphine tolerance. This finding confirms the first report of agmatine’s ability to prevent the development of chronic tolerance to systemically administered morphine (Kolesnikov et al., 1996) and extends this previous result to include a spinal site of action.

Our study is composed of four experiments using two NMDA receptor antagonists (MK801, LY235959), one accepted modulator of NOS (7-NI) and one agent (agmatine) that may affect NOS. The observations presented consistently implicate the NMDA receptor/NOS cascade in the development of acute tolerance to intrathecally administered morphine. Each of these experiments produced results comparable to those reported previously using chronic models of morphine tolerance. That the outcomes of our studies examining acute tolerance correlate with outcomes from comparable studies of chronic morphine tolerance suggests that these two phases of tolerance share underlying mechanisms. This is the case for those agents that modulate the NMDA receptor/NOS cascade in both morphine and ethanol tolerance (Khanna et al., 1992, 1993, 1994; Rafi-Tari et al., 1996; Wu et al., 1993) and may hold true for other receptor systems that participate in the development of tolerance. If so, the acute tolerance paradigm carries powerful predictive experimental value.

We propose that acutely induced spinal tolerance offers several advantages for future studies of the mechanisms of opioid tolerance. The acute tolerance paradigm reflects improvements in experimental design including reduced tolerance level at time of probe test, improved general health of the animal, increased potential for data collection due to simplified animal protocols, decreased variability between subjects and decreased experimental time. Acute tolerance also favors mice as subjects because mice are the most appropriate subjects for acute spinal injection. Quantitative pharmacological investigations of receptor/effect-mechanisms in vivo require large numbers of experimental subjects for which mice are economically practical. Furthermore, gene-targeted mouse lines, engineered to selectively disrupt the expression or function of individual receptor subtypes or signal transduction systems, present excellent systems for determining the molecular mechanisms of spinal tolerance. Current lines potentially useful for studies of opioid antinociception include gene-targeted mice with altered \( \alpha_{2A} \) receptors (MacMillan et al., 1996), absent \( \alpha_{2B} \) or \( \alpha_{2C} \) adrenergic receptors (Link et al., 1996), absent PKC\( \gamma \) (Abeliovich et al., 1993), absent neuronal NOS (Huang et al., 1994) and absent transcription factor CREB (Maldonado et al., 1996). However, a frequently mentioned concern about the application gene-targeted animals to studies of mechanism in vivo is the potential for compensatory changes during development; these are difficult to identify and may limit interpretation of the data. A complementary technique recently applied to studies of antinociception is the central administration of antisense oligodeoxynucleotides to selectively disrupt expression of specific receptor subtypes (Bilsky et al., 1996b; Lai et al., 1996; Standifer et al., 1994). The repeated i.t. administration of oligodeoxynucleotides implicit in these experiments would likely confound the interpretation of chronic tolerance studies. However multiple oligodeoxynucleotide pretreatments preceding a single injection acute tolerance study may prove very effective. We propose that use of the acute spinal tolerance induction method in gene-targeted mouse lines and in antisense-pretreated mice will facilitate investigations of tolerance at a molecular level at minimal cost and with limited experimental confounds. Acute tolerance is also advantageous because the brevity of agonist exposure permits evaluation of the critical time points in tolerance induction, i.e., the upslope, peak and downslope. Therefore, the opportunity arises to explore temporal characteristics of induction of tolerance, which may not be resolvable with chronic models. Finally, incorporation of the acute tolerance paradigm can improve the design of electrophysiological studies, enabling comparisons within the same neuron between the nontolerant and tolerant states, each cell serving as its own control. In vitro electrophysiological evidence supports the proposal that acute tolerance parallels chronic tolerance (Fiorillo and Williams, 1996).

Although there exist a substantial number of investigations using the acute tolerance paradigm, a very limited number of studies compare characteristics of acute morphine tolerance to those observed in similarly designed chronic studies. Several groups have observed comparable results between acutely and chronically induced morphine tolerance (Huidobro-Toro and Way, 1978; Narita et al., 1995; Yano and Takemori, 1977). Interestingly, comparable results have also been observed in models investigating acute and chronic ethanol tolerance, specifically when the tolerance induction is accompanied by repeated synaptic activation (Le et al., 1992). The fact that opioid receptor activation can lead to activation of PKC and facilitation of NMDA receptor function (Chen and Huang, 1992) provides a possible substrate for such a pairing of opioid receptor occupation and repeated synaptic activation. In agreement with this idea, our study has demonstrated that, with respect to the NMDA receptor/NOS cascade, acute morphine tolerance can be manipulated similarly and yield results comparable to those of previous studies of chronically induced morphine tolerance.

This report is not the first to suggest that acute and chronic morphine tolerance may be mechanistically similar. Schmidt and Livingston (1933a, b, c) reported that the induction of acute systemic tolerance to the vasodilatory and convulsant effects of morphine in dogs paralleled results from their similarly implemented chronic studies. Based on their findings, Schmidt and Livingston (1933a, b, c) speculated that the cellular mechanisms underlying both phenomena may be closely related. Subsequent work (Huidobro-Toro and Way, 1978) agreed with but did not fully explore that proposal. Our study reexamines that question in terms of spinal analgesic systems and during a time when technological advances facilitate higher resolution investigations.

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