Spinal Amino Acid Release and Precipitated Withdrawal in Rats Chronically Infused with Spinal Morphine

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Glutamate receptors are implicated in the genesis of opioid tolerance and dependence. Factors governing release of amino acids in systems chronically exposed to opiates, however, remain undefined. Using rats, each prepared with a spinal loop dialysis catheter and with a chronic lumbar intrathecal infusion catheter connected to a subcutaneous minipump, the release of amino acids before and during antagonist-precipitated withdrawal in unanesthetized rats was examined. Spinal infusion of morphine (20 nmol/ μ l/hr) for 4 d had little effect on resting release of amino acids. In morphine-infused, but not saline-infused, rats naloxone (2 mg/kg, i.p.) evoked an immediate increase in the release of L-glutamate ($299 \pm 143\%$) and taurine ($306 \pm 113\%$) but not other amino acids. The magnitude and time course of the release of these amino acids significantly correlated with behavioral indices of withdrawal intensity. Acute

intrathecal pretreatment immediately before naloxone with clonidine (20 μ g; α_2 agonist), MK-801 (3 μ g; noncompetitive NMDA antagonist), or aminophosphonopentanoic acid (AP-5; 3 μ g; competitive NMDA antagonist) suppressed naloxone-induced increases in spinal L-glutamate and taurine release and behavioral signs of withdrawal in spinal morphine-infused rats. Results point to a correlated increase in spinal L-glutamate release, which contributes to genesis of the opioid withdrawal syndrome. Agents such as clonidine that suppress opioid withdrawal may owe their action to an inhibition of excitatory amino acid release. The effects of MK-801 and AP-5 suggest a glutamate-evoked glutamate release.

Key words: spinal; morphine; tolerance; MK-801; AP-5; clonidine; withdrawal

Continuous infusion or repeated spinal injections of opiates result in an analgesic effect that disappears over 4–7 d, despite the continued steady-state concentration of the drug (Yaksh et al., 1977; Advokat et al., 1987; Stevens and Yaksh, 1989a,c, 1992; Sosnowski and Yaksh, 1990; Russell et al., 1987), e.g., tolerance. In this state, naloxone then evokes an increase in autonomic/motor activity and a hyperalgesic state (Stevens and Yaksh, 1989b). As with studies using systemic delivery (Rasmussen et al., 1991; Tanganelli et al., 1991; Trujillo and Akil, 1991; Higgins et al., 1992), co-injection of NMDA receptor antagonists will do the following: (1) prevent reduction in antinociceptive activity (Dunbar et al., 1994; Mao et al., 1994), (2) reduce the right shift in the dose–response curve otherwise observed in chronically infused rats (Dunbar et al., 1994); and (3) attenuate precipitated withdrawal (agitation and hyperalgesia) evoked by naloxone in rats previously exposed to spinal opiates (Dunbar et al., 1994; Mao et al., 1994). Enhanced activity of excitatory amino acid (EAA) transmission may thus contribute to the genesis of opioid tolerance and mediate the withdrawal signs in spinally dependent animals. In the brain, naloxone-precipitated opioid withdrawal is associated with hyperactivity of locus coeruleus neurons (Rasmussen et al., 1990). Electrophysiological (Akaoka and Aston-Jones, 1991) and voltametric (Hong et al., 1993) studies indicate that this response

can be attenuated by EAA antagonists. Microdialysis studies in anesthetized morphine-tolerant rats show that naloxone evokes an enhanced glutamate and aspartate release in locus coeruleus (Aghajanian et al., 1994).

In the spinal cord, releasable pools of EAA are found in opioid-sensitive primary afferent neurons (Merighi et al., 1991; Tracey et al., 1991). NMDA receptors exist in spinal dorsal horn (Furuyama et al., 1993; Liu et al., 1994) and were identified on primary afferents (Liu et al., 1994), suggesting a positive feedback loop. Acute or chronic spinal delivery of NMDA or kainic acid evokes hyperalgesia and tactile allodynia (Coderre and Melzack, 1992; Malmberg and Yaksh, 1992) and increases spinal EAA release *in vivo* (Sorkin, 1993; Marsala et al., 1995a,b).

The observations outlined above suggest four hypotheses. (1) As concurrent, but not acute, spinal NMDA antagonism attenuates development of opioid tolerance with continued or repeated opiate exposure, then chronic opioid receptor activation leading to tolerance may induce or require an ongoing increase in spinal EAA release. (2) As precipitated opiate withdrawal yields a state of hyperalgesia that is NMDA antagonist-sensitive, then naloxone in an animal rendered spinally tolerant to morphine should evoke an increase in the spinal release of EAAs. (3) As NMDA receptor activation evokes spinal glutamate release, spinal antagonism of NMDA receptors should inhibit naloxone-evoked glutamate release. (4) α_2 agonists reduce spinal glutamate release (Kamisaki et al., 1993a), and α_2 agonists/glutamate receptor antagonists attenuate opiate withdrawal signs (Milne et al., 1985; Tanganelli et al., 1991). Accordingly, we propose that spinal clonidine or NMDA antagonism will reduce the withdrawal-correlated release of spinal glutamate.

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MATERIALS AND METHODS

All work described in this study was performed according to protocols approved by the Institutional Animal Care and Use Committee of the University of California, San Diego. In the present studies, we used unanesthetized rats prepared with chronic lumbar intrathecal catheters with subcutaneously placed osmotic pumps for drug delivery (Yaksh and Stevens, 1986) and chronic lumbar loop dialysis catheters (Marsala et al., 1995a,b) to measure ongoing EAA release.

Continuous intrathecal infusions. Experiments were performed on male Sprague–Dawley rats (400–450 gm) maintained on a 12 hr light/dark cycle in individual cages and given full access to food and water. Under halothane anesthesia, animals were implanted with an intrathecal catheter attached to a preloaded osmotic minipump. The intrathecal catheter was prepared by making a U-bend in the polythene tubing (Intramedic PE-10) and pulling the “U”-section through a piece of SILASTIC tubing to form a small loop (Sosnowski and Yaksh, 1990). The catheter end to be implanted in the intrathecal space was stretched to reduce its diameter. This stretched catheter was then inserted through a slit in the cisterna magna such that its tip lay at the lumbar enlargement. The free end of the catheter was attached to the osmotic minipump via a connector prepared from PE-60 polyethylene tubing. The U-portion of the catheter was externalized to protrude from the skin on top of the skull. The pump was inserted under the skin on the back of the neck.

The rats received infusion at the rate of 1 μ l/hr of either saline (0.9%) or morphine sulfate solution (6.7 mg/ml). This treatment was chosen based on previous work that indicated that this concentration would yield an acute, near-maximal increase in the hot-plate response latency and produce a significant dependence as revealed by a prominent naloxone-induced withdrawal (Stevens and Yaksh, 1989a,b; Sosnowski and Yaksh, 1990).

Insertion of the microdialysis catheter. The intrathecal loop dialysis catheter for dialysis was constructed using cuprophane hollow fibers (Filtral, AH 69-HF) as described previously (Marsala et al., 1995a,b). In brief, an 18 cm length of fiber was formed into a loop with the arms of the loop coated to block permeability, except for the 4 cm portion that constituted the loop (active dialysis site). The loop section was inserted through the cisternal slit, taking extreme care to avoid contact with spinal roots (indicated by appearance of a skin twitch). The two free ends of the dialysis loop catheter were glued to 4 cm lengths of PE-10 tubing that were then externalized through the skin at the top of the skull. Each arm of the PE-10 tubing was plugged with stainless steel pins. All of the wounds were sutured, and the animal returned to its home cage after surgery. Animals showing visible forelimb or hindlimb motor deficits after recovery were killed.

Intrathecal microdialysis. Spinal microdialysis was carried out on the fourth day after surgery to assess amino acid release immediately before and after a single naloxone challenge to precipitate the opioid withdrawal response. To accomplish the dialysis, the two ends of the microdialysis catheter were first connected to polyethylene (PE-50) tubing to serve as inflow and outflow tubes. The rat was then transferred to a clear circular Plexiglas chamber. Perfusion was initiated with previously aerated artificial CSF (ACSF) at the rate of 10 μ l/min. The composition of ACSF was (in mM): 151.1 Na⁺, 2.6 K⁺, 0.9 Mg²⁺, 1.3 Ca²⁺, 122.7 Cl⁻, 21 HCO₃⁻, 2.5 HPO₄, and 3.5 dextrose. The solution was bubbled with 95% O₂/5% CO₂, and the final pH was adjusted to 7.2. The outflow tubing was placed in an Eppendorf tube, and the dialysate was collected on ice. Dialysate samples were frozen at -20°C until analysis by HPLC.

To assess the effect of chronic morphine infusion on the baseline glutamate release during the development of morphine tolerance (i.e., 1–3 d after activation of miniosmotic pump), an additional group of animals was used. In those animals, IT loop catheter and IT PE-10 catheter were implanted as described. Two days after implantation and 30 min washout period, two baseline samples (10 min duration each) were collected. After this, the miniosmotic pump loaded with either morphine ($n = 8$) or saline ($n = 7$) was connected to the single IT catheter. After pump implantation at days 1, 2, and 3, the hot-plate response latency was measured followed by collection of dialysate samples.

Assessment of antinociception. Before surgery, the latency of the hind-paw lick response was determined using the hot-plate test (temperature 52.5°C). In the absence of a response, the test was terminated at 60 sec (cutoff) and that latency assigned. On successive days after surgery, the response latency was determined once daily (between 8:00 and 10:00 A.M.). All animals were weighed once daily and inspected for the presence of motor deficits. Rats showing deficits were excluded from the study.

Induction of opioid withdrawal. After a 30 min washout period, two samples of the dialysate (controls) were collected at 10 min intervals. These constitute the day 4 control sample. After the second sample collection, the animal was given a single injection of naloxone (2 mg/kg, i.p.) (morphine-infused animals, $n = 7$; saline-infused animals, $n = 5$). Perfusion was continued, and three dialysate samples were collected at 10 min intervals. After this, two additional samples were collected at 15 min intervals.

During the microdialysis session that followed the 30 min washout period, the animals were observed for the presence of withdrawal signs: allodynia (light touch, air puff), spontaneous vocalization, abnormal body posture, ejaculation, urination, diarrhea, exophthalmos, piloerection, headshakes, hindlimb extension, chewing and licking response, and tremor. The intensity of signs elicited was recorded using a rating scale of one to three (mild, moderate, and severe) on a standardized score sheet (Milne et al., 1985; Cridland et al., 1991). The total withdrawal score attained after naloxone was calculated by adding the individual scores.

In experiments involving the effects of intrathecal clonidine (20 μ g; $n = 9$), MK-801 (3 μ g; $n = 4$), or AP-5 (3 μ g; $n = 7$) effects on naloxone-precipitated withdrawal, the drug was injected intrathecally through the polyethylene infusion catheter after cutting the previously exteriorized loop. The drug injection (10 μ l) was followed by a saline flush (10 μ l). The dialysate sample during the 10 min period after the drug injection was discarded, and collection resumed. After this period, the animal was given a single naloxone injection (2 mg/kg i.p.), and dialysate samples were collected as described above. The doses of NMDA antagonists were chosen because they represented the minimum doses that produce a significant antagonism of the second phase of the formalin test, the thermal hyperalgesia of Bennett, as well as the tactile allodynia of the Chung model of neuropathy (Yamamoto and Yaksh, 1992a,b; Chaplan et al., 1993) (S. Chaplan, A. Malmberg, and T. Yaksh, unpublished observations), all models believed to have significant NMDA receptor-mediated components (Yaksh, 1993b). The dose of clonidine was chosen based on its ability to produce a thermal analgesia (Takano and Yaksh, 1992) and to block the tactile allodynia produced in a neuropathic pain model (Yaksh et al., 1995). In all cases, the doses selected had been shown to have no effect on motor function after spinal delivery.

After the last dialysate collection, the inflow/outflow catheters were disconnected and the animal returned to its home cage. On the next day, the animal was deeply anesthetized and perfused with saline followed by 10% formalin. Twenty-four hours later, the spinal cords were removed from the vertebral column, and the placement of the infusion catheter and dialysis catheter in the intrathecal space was verified.

Drugs. Clonidine HCl (Research Biochemicals, Natick, MA), MK-801 (Research Biochemicals), AP-5 (Research Biochemical), morphine sulfate (Merck, Darmstadt, Germany), and naloxone HCl (DuPont NEN, Rahway, NJ) were mixed in sterile saline (0.9%, USP) in polyethylene vials immediately before use.

Amino acid analysis. The dialysis samples were analyzed for different amino acids by the phenylisothiocyanate derivatization procedure (Cohen and Strydom, 1988) using a Waters HPLC equipped with a reverse-phase C-18 column (3.9 $\times$ 300 mm², 4 mm particle) and ultraviolet detector (Waters, Milford, MA). Methionine sulfone was added to each sample as an internal standard. External standards (40, 400, and 4000 pmol of authentic amino acids) were run at the beginning and the end of each sample group. Peak heights were normalized to the methionine sulfone peak and then quantified based on a linear relationship between peak height and amounts of corresponding standards. Sensitivity was 5–10 pmol/injection.

Data expression and statistics. The release during each period after naloxone was expressed as percent baseline. Statistical analysis of biochemical and behavioral results was carried out with one-way repeated-measures ANOVA followed by appropriate post hoc tests (Fisher, Scheffe, and Dunnett). Examination of the covariance between behavior and release was accomplished using linear regression analysis plotting the behavioral score during withdrawal versus the release for each individual animal, and the regression was calculated using StatView (Abacus, Calabasas, CA). $p < 0.05$ was considered significant.

RESULTS

Development of antinociceptive tolerance

Continuous intrathecal infusion of morphine (20 nmol/ μ l/hr) resulted in a significant near-maximal increase in the hot-plate response latency on days 1 and 2. Thereafter, the latency dis-

Figure 1. Hot-plate response latency in rats receiving continuous spinal infusion of saline ($n = 5$; open circles) or the infusion of morphine (20 nmol/ μ l/hr; $n = 27$; filled circles). On day 4, the rats were entered into the study to examine the effects of naloxone. Each line presents the mean $\pm$ SEM of 27 and 5 rats, respectively.

played a progressive decline in effect over the ensuing days (Fig. 1). No evidence of motor dysfunction was noted in these rats at any time. Saline-infused animals showed no change in the hot-plate response latency over the interval of infusion.

Spinal amino acid release

Table 1 shows the baseline release of glutamate, taurine, glycine, serine, and glutamine in all treatment groups. There was no significant difference in baseline release at day 4 among the infusion treatment groups. Aspartate levels were typically at or near detection limits under control conditions.

The injection of naloxone (2 mg/kg, i.p.) in saline-infused animals failed to influence baseline release of any amino acid. For clarity, Figure 2 displays the time course of postnaloxone levels for the concentrations of glutamate, taurine, and glycine in the spinal dialysate. In marked contrast, the injection of antagonist in the morphine-infused animals provoked an immediate twofold increase in glutamate and taurine release. Whereas release of glutamate very rapidly returned to control level after the naloxone injection, taurine release showed a delayed decline to baseline levels over the ensuing 30 min interval (Fig. 2).

The concentrations of several other amino acids including serine, asparagine, glutamine, and citrulline could be detected under resting conditions, and these amino acids showed no change in concentrations after naloxone.

Effect of morphine infusion on basal spinal glutamate release

Chronic infusion of morphine had no significant effect on the baseline glutamate release compared with baseline pre-morphine infusion levels (collected 2 d after surgery, immediately before initiation of morphine infusion). These values were as follows: day 1 = $110 \pm 8\%$; day 2 = $101 \pm 7\%$; day 3 = $99 \pm 11\%$ ($n = 8$). Comparably, no significant changes in the concentration of several other amino acids (taurine, serine, aspartate) were observed. As in morphine-infused animals, 3 d infusion of saline had no significant effect on tonic glutamate release, and the levels remained unchanged for 3 d of infusion: day 1 = $107 \pm 8\%$; day 2 = $108 \pm 7\%$; day 3 = $106 \pm 16\%$ ($n = 7$).

Behavior

The withdrawal scores, representing naloxone-induced behavioral and autonomic hyperactivity, are shown in corresponding histograms in Figure 2. In the morphine-infused group, naloxone provoked a brisk and immediate withdrawal response involving profound allodynia to light touch or air puff, abnormal body posture indicative of a writhing response, spontaneous vocalization, and autonomic hyperactivity (indicated by urination, diarrhea, ejaculation, piloerection, and exophthalmos). Some animals also showed head shakes, tremor, and extension of hindlimbs. The highest withdrawal scores were obtained during the first 10 min after the antagonist injection, a time period that corresponded to the greatest release of glutamate and taurine. Both spinal amino acid release and the withdrawal scores declined over time after antagonist injection, with the behavioral scores remaining slightly elevated for the duration of the experimental period.

Acute intrathecal AP-5, MK-801, and clonidine

In separate groups of morphine-infused rats, the effects of the spinally administered α_2 agonist clonidine, noncompetitive NMDA antagonist MK-801, and competitive NMDA antagonist AP-5 on both the amino acid release response and the behavioral withdrawal signs elicited by naloxone were evaluated.

Intrathecal injection of the noncompetitive NMDA antagonist MK-801 (3 μ g/10 μ l) 10 min before naloxone had no effect on resting amino acid release, but it prevented the increase in amino acid release and reduced withdrawal scores. However, unlike clonidine, which suppressed almost all signs of withdrawal (including allodynia; see below), MK-801 appeared to exert an incomplete suppression of several of the components of the withdrawal index (see Fig. 3). Thus, the rats given this agent showed lack of allodynia, abnormal postures, ejaculation, and spontaneous vocalization; however, they continued to show mild autonomic hyperactivity (diarrhea, reduced spontaneous mobility, and exophthalmos). MK-801-treated rats showed no signs of sedation. No effects on motor function were observed.

Pretreatment with the competitive NMDA antagonist AP-5 (3 μ g/10 μ l) provided effects similar to those seen in MK-801-treated animals. Thus, it had no effect on resting amino acid release and resulted in only modest (20–35%) naloxone-evoked amino acid release, and there was a significant suppression of behavioral signs of withdrawal. As in the MK-801 treatment group, all AP-5-treated animals showed a lack of allodynia, abnormal postures, ejaculation, and spontaneous vocalization. No effects on motor function were observed.

Intrathecal clonidine (20 μ g/10 μ l) given 10 min before naloxone challenge had no effect on resting amino acid release, but it abolished the increase in glutamate and taurine release elicited by the antagonist in the morphine-treated group (see Fig. 4). During the last two dialysate collection periods, the concentrations of glutamate fell below the baseline values ($p < 0.05$). Glycine concentration was significantly below baseline between 20 and 60 min after naloxone injection ($p < 0.05$). Intrathecal clonidine resulted in a near complete elimination of the behavioral and autonomic signs precipitated by naloxone injection. During this interval, the animals displayed no evidence of sedation or motor dysfunction, but after 45–60 min some signs of behavioral depression were noted.

Table 1. Baseline release in all experimental groups (pmol/tube)

Experimental group	Saline (<i>n</i> = 5)	Morphine (<i>n</i> = 7)	Morphine + clonidine (<i>n</i> = 9)	Morphine + MK-801 (<i>n</i> = 4)	Morphine + AP-5 (<i>n</i> = 7)
Glutamate	45 (14–72)	59 (22–94)	56 (37–87)	59 (29–96)	50 (42–76)
Taurine	109 (44–173)	215 (107–379)	212 (134–304)	127 (44–209)	128 (92–150)
Glycine	322 (95–543)	728 (376–1319)	535 (332–675)	311 (198–474)	521 (352–593)
Serine	235 (178–310)	403 (215–653)	297 (217–381)	381 (197–486)	299 (234–340)
Glutamine	1401 (1061–2017)	1746 (1156–2543)	1649 (1521–2009)	2527 (1195–3518)	1871 (1391–2595)

Data are expressed as a median with 95% confidence intervals.

Correlation between spinal amino acid release and withdrawal behavior

Figure 5 indicates the relationship between the release of glutamate or taurine and the total withdrawal scores obtained in each animal of all treatment groups used in this study. As indicated, there was a highly significant covariance between the magnitude of glutamate and taurine release during the 20 min period after naloxone (0–10 and 10–20 min epochs) and the prominence of the precipitated withdrawal as indicated by the withdrawal scores. Thus, as indicated by the regression, clonidine- and AP-5/MK-801-treated groups displayed correlated reductions in both the severity of the naloxone-induced behavior and spinal amino acid

release. To demonstrate the selectivity of this correlation, glycine concentrations in spinal dialysates displayed no correlation with withdrawal scores.

Localization of spinal catheters

After completion of these studies, necropsy was accomplished, which indicated that all loop catheters had some of their active dialysis portion located at the level of the lumbar (L1–L3/4) spinal cord. The infusion catheter tip was reliably found to be located at the level of the L1/L2 spinal cord. In the majority of spinal cords, the loop catheter was localized on the dorsal or dorsolateral part of the spinal cord, whereas in some, both profiles of the dialysis

Figure 2. Changes in glutamate, taurine, and glycine concentrations (% of baseline) in spinal microdialysate and corresponding withdrawal score after naloxone (2 mg/kg, i.p.) injection in rats having been spinally infused for 4 d with morphine (20 nmol/ μ l/hr; *n* = 7) or saline (*n* = 5). Note a significant release of glutamate and taurine in the initial 10 min after naloxone injection in morphine-pretreated animals. Data are expressed as mean $\pm$ SEM of the percent of baseline release. Baseline levels of release are presented in Table 1 (AAs release: one-way ANOVA for multiple comparisons: morphine/naloxone/glutamate, *F* = 3.8; taurine, *F* = 5.4; glycine, *F* = 9.7; saline/naloxone/glutamate, *F* = 1.2; taurine, *F* = 2; glycine, *F* = 2.1) (withdrawal: morphine/naloxone, *F* = 64; saline/naloxone, *F* = 4.6) (**p* < 0.05 compared with pre-naloxone values).

Figure 3. Changes in glutamate, taurine, and glycine concentrations (% of baseline) in spinal microdialysate and corresponding withdrawal score after naloxone injection (2 mg/kg, i.p.). Rats received pretreatment with intrathecal AP-5 (3 μ g/10 μ l, left, n = 7) or MK-801 (3 μ g/10 μ l, right, n = 4). As indicated, this pretreatment resulted in a near complete suppression of the observed withdrawal behavior and the naloxone-precipitated glutamate and taurine release, as otherwise observed in Figure 2. Data are expressed as mean $\pm$ SEM of the percent of baseline release. Baseline levels of release are presented in Table 1 (AAs release: one-way ANOVA for multiple comparisons: morphine/AP-5/naloxone/glutamate, F = 2.1; taurine, F = 4.6; glycine, F = 2.2; morphine/MK-801/naloxone/glutamate, F = 0.88; taurine, F = 0.9; glycine, F = 0.98) (withdrawal: morphine/AP-5/naloxone, F = 3.7; morphine/MK-801/naloxone, F = 6.8) (* p < 0.05 compared with pre-naloxone values).

fiber were found to be adjacent to the ventral horn. Comparison of the levels of amino acid release in the several animals showed no consistent differences related to the disposition of these dialysis probes. Examination of the location of the intrathecal infusion catheter revealed them to be reliably adjacent the one of the arms of the loop catheter at a distance of no more than 1–2 mm.

DISCUSSION

Activation of spinal terminals associated with bulbospinal pathways, primary afferent and intrinsic neurons, results in a stimulus-dependent increase in the concentrations in the lumbar spinal CSF in rat and cat of neurotransmitters specific for those terminals, e.g., noradrenaline/serotonin (Tyce and Yaksh, 1981), enkephalin/neurotensin (Yaksh and Elde, 1980, 1981; Yaksh et al., 1982b), cholecystokinin/vasoactive intestinal polypeptide (Yaksh et al., 1982a), and substance P (Jhamandas et al., 1984; Go and Yaksh, 1987; Aimone and Yaksh, 1989). In the unanesthetized rat with the loop dialysis catheter, hindpaw formalin (Sanghvi and Yaksh, 1994; Malmberg and Yaksh, 1995; Marsala et al., 1995a,b) and spinal injection of EAA (Marsala et al., 1995a,b) increase spinal extracellular glutamate/taurine concentrations. Thus, concentrations of analyte in the intrathecal space covary predictably with activity of the respective terminals.

In the present study, naloxone delivered systemically to the rat exposed to 4 d of morphine infusion evoked a reliable, time-dependent increase in dialysate glutamate and taurine concentrations in the unanesthetized rat. Failure to observe amino acid

release with naloxone in nontolerant animals, and the effect on some (glutamate and taurine) but not other amino acids (e.g., glycine), argues against a “nonspecific” effect mediated via altered dialysis function. Alternatively, the increased release may reflect motor activity otherwise observed during withdrawal. Although possible, we note that glycine levels did not change. Glycine plays a major role in Renshaw cell circuitry, which is routinely activated during motor function (Todd and Sullivan, 1990; Schneider and Fyffe, 1992). Moreover, in other studies, after injection of formalin into the hindpaw, there is a biphasic appearance of extensive flinching and jerking of the limb of the injected paw. Glutamate and aspartate release, however, occurs only during phase I (Malmberg and Yaksh, 1995; Marsala et al., 1995a,b). Although not absolute, this dissociation of spinal EAA release and activity between phase 1 and phase 2 indicates that significant increases in motor activity per se are not associated with changes in spinal EAA release. Accordingly, in the present studies we do not believe that the increased spinal EAA release is secondary to the motor behavior observed during withdrawal.

Spinal tolerance and glutamate receptor activity

Co-spinal administration of NMDA antagonists attenuates the loss of opiate effects with daily spinal morphine injection (Mao et al., 1994) or infusion (Dunbar and Yaksh, 1994). In addition, withdrawal and hyperalgesia induced by naloxone in spinal morphine tolerant rats are attenuated by spinal delivery of an NMDA receptor antagonist (Dunbar and Yaksh, 1994; Mao et al., 1994).

Figure 4. Changes in glutamate, taurine, and glycine concentrations (% of baseline) in spinal microdialysate and corresponding withdrawal score after naloxone injection (2 mg/kg, i.p.). Rats received pretreatment with intrathecal clonidine (20 μ g/10 μ l, $n = 9$). We observed significant suppression of the observed withdrawal behavior and near complete block of the naloxone-precipitated glutamate and taurine release, as otherwise observed in Figure 2. Data are expressed as mean $\pm$ SEM of the percent of baseline release. Baseline levels of release are presented in Table 1 (AAs release: one-way ANOVA for multiple comparisons: morphine/clonidine/naloxone/glutamate, $F = 3.9$; taurine, $F = 8.1$; glycine, $F = 9.8$) (withdrawal: morphine/clonidine/naloxone, $F = 17$) (* $p < 0.05$ compared with pre-naloxone values).

These observations are in accord with behavioral hyperalgesia being induced by intrathecal NMDA (Malmberg and Yaksh, 1992). The observations that NMDA antagonists prevent onset of tolerance and acutely prevent the appearance of withdrawal in the morphine tolerant animal suggest that chronic opiate exposure leads to an enhancement in spinal glutamatergic transmission. These release levels would be increased additionally during precipitated withdrawal. We observed, however, that the chronic infusion of morphine did not lead to an increase in glutamate release. It might be argued that the site of release induced by the continuous opiate action is not adequately sampled or that the release evoked by morphine is modest, but physiologically important (see Marsala et al., 1995a,b). Nevertheless, physiological stimuli (e.g., afferent activation by inflammation of the knee joint or formalin in the paw) evoke readily detectable increases in spinal dialysate concentrations (Malmberg and Yaksh, 1995; Yang et al., 1995). This suggests that the role of the NMDA receptor in the development of tolerance does not relate to an enhanced level of glutamate release.

Spinal dependence/withdrawal and glutamate release

Naloxone injection evoked agitation and hyperalgesia as well as a concurrent increase in spinal glutamate release in morphine-, but not saline-infused, rats. The lack of naloxone effect on behavior

Figure 5. Correlation between the mean $\pm$ SEM glutamate, taurine, and glycine release during the initial 20 min (0–10 and 10–20 min samples) after naloxone injection and withdrawal score in all experimental groups (saline alone; morphine alone; morphine + MK-801; morphine + AP-5; morphine + clonidine) (individual release data vs withdrawal scores were correlated with Spearman–Rank correlation: glutamate, $r = 0.46$, $p = 0.001$; taurine, $r = 0.45$, $p = 0.002$; glycine, $r = -0.04$, $p = 0.8$). Glutamate and taurine correlations with withdrawal index were highly significant. As in the case of glycine (bottom), no significant correlation between the magnitude of other amino acids (Asp, Ser, Glu, Asp) and the withdrawal score was found (data not shown).

and release in saline-infused animals suggests that animal handling and injection procedures, likely activating primary afferent input into the spinal cord, do not contribute to the release or behavior seen in morphine-infused animals. These results in spinal cord are similar to those in which naloxone augmented glutamate release from locus coeruleus in morphine-tolerant anesthetized rats (Aghajanian et al., 1994).

Mechanisms of spinal glutamate release and opiate withdrawal

Continued exposure of the spinal cord to an opiate induced a state in which naloxone resulted in an enhanced spinal release of glutamate. The release by naloxone after chronic spinal opiate exposure may reflect the withdrawal of opiate receptor occupancy regulating glutamatergic terminal activity. Glutamate is located in nociceptive and non-nociceptive primary afferents (Broman et al., 1993) and is released from spinopetal (Potashner and Dymczyk, 1986; Benson et al., 1991) and intrinsic interneurons (Paleckova et al., 1992). Presynaptic opioid receptors inhibit transmitter release from C fibers (Yaksh, 1993a), and morphine reduces spinal glutamate release evoked by paw formalin in nontolerant rats (Malmberg and Yaksh, 1995). Opiate-binding sites postsynaptic to the primary afferent may interact with glutamatergic neurons. Spinobulbar projections have been identified as being glutamatergic (DeBiasi and Rustoni, 1990), and spinopetal neurons can be inhibited by opiates (see Yaksh, 1993a).

Although naloxone had no effect on release in the saline-infused rat, low concentrations of opiates in dorsal root ganglion cells may enhance depolarization through a G_s -coupled protein (Crain and Shen, 1992; Shen and Crain, 1992, 1994). With chronic opiate exposure, this response displays sensitization, and naloxone acquires the ability to unmask the excitatory effect. Thus, the evoked release observed in the present study with naloxone may reflect such a direct action in a system sensitized by chronic opiate exposure.

Spinal $\alpha 2$ and NMDA antagonism on release and withdrawal

The $\alpha 2$ agonist clonidine attenuates opiate withdrawal in humans (Gold, 1978) and, after systemic and spinal delivery, in animals (Aghajanian, 1978) (this study). Spinal clonidine reduces spinal opiate withdrawal signs. Previous work has shown that, like μ opiates, $\alpha 2$ adrenoreceptor agonists can hyperpolarize dorsal horn neurons and act presynaptically on primary afferents to reduce C fiber neurotransmitter release (Yaksh et al., 1993) and diminish spinal glutamate release (Kamisaki et al., 1993a). At the spinal level, the blockade of the hyperalgesia and the glutamate release by spinal clonidine may reflect a combined presynaptic effect preventing the release of glutamate, as well as a postsynaptic action to hyperpolarize neurons that are otherwise depolarized by the removal of the opiate.

With regard to the NMDA antagonist, MK-801 attenuates opioid withdrawal signs (Rasmussen et al., 1991, 1992; Teganelli et al., 1991; Higgins et al., 1992) (but see Kreeger et al., 1994). The ability of two NMDA receptor antagonists (MK-801 and AP-5) to diminish the agitation and withdrawal signs is consistent with the powerful agitation and allodynia produced by intrathecal NMDA (Malmberg and Yaksh, 1992) and the naloxone-evoked release of spinal glutamate observed in the present studies. The ability of NMDA antagonism to diminish spinal release of glutamate corresponds with the observation that intrathecal injection of NMDA or kainic acid increases spinal glutamate release (Sorkin, 1993; Marsala et al., 1995a,b). This suggests a glutamate-evoked glutamate release. Such release may occur from spinal neurons postsynaptic to the primary afferent and is consistent with the ability of glutamate to excite spinal neurons (Aanonsen et al., 1990). Alternatively, glutamate might evoke release from the primary afferent, as

suggested by the presence of NMDA receptors on primary afferent terminal (Liu et al., 1994).

Spinal taurine release

In brain, NMDA evokes taurine release (Lehman et al., 1984). Taurine release occurring during opioid withdrawal, therefore, could be initiated by factors governing glutamate release. Although the role played by spinal taurine on spinal function during withdrawal is not known, (1) taurine will inhibit depolarization-evoked release of glutamate from brain synaptosomes (Kamisaki et al., 1993b), and (2) intrathecal taurine blocks biting and scratching behavior evoked by injection of SP but not by NMDA or non-NMDA receptor agonists (Smullin et al., 1990). Such results suggest that taurine buffers spinal EAA release and, consequently, the behavioral hyperactivity associated with glutamate receptor activation.

In conclusion, the present studies point to an enhanced spinal release of glutamate during precipitated spinal opioid withdrawal, a finding in parallel with the ability of increased spinal glutamate receptor activity to induce agitation and hyperalgesia. Additional correlative support arises from the ability of spinal $\alpha 2$ agonists and two NMDA receptor antagonists to suppress concurrently spinal opioid withdrawal and spinal excitatory amino acid release. Aside from the insights provided regarding withdrawal and glutamate release, these results suggest several provocative hypotheses. One such hypothesis is that if spinal glutamate (NMDA) receptor occupancy enhances tolerance and if naloxone evokes spinal glutamate release in a morphine-exposed animal, then periodic withdrawal of opiate receptor occupancy may result in periodic increases in glutamate release and an augmentation of the tolerance-dependence state produced by a given degree of morphine exposure. We have recently confirmed this hypothesis (Ibuki et al., 1995).

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