Behavioral/Systems/Cognitive

Nonopioid Actions of Intrathecal Dynorphin Evoke Spinal Excitatory Amino Acid and Prostaglandin E_2 Release Mediated by Cyclooxygenase-1 and -2

Lee Koetzner,[1] Xiao-Ying Hua,[1] Josephine Lai,[3] Frank Porreca,[3,4] and Tony Yaksh[1,2]

Departments of [1]Anesthesiology and [2]Pharmacology, University of California, San Diego, La Jolla, California 92093, and Departments of [3]Pharmacology and [4]Anesthesiology, University of Arizona, Tucson, Arizona 85724

Spinal dynorphin is hypothesized to contribute to the hyperalgesia that follows tissue and nerve injury or sustained morphine exposure. We considered that these dynorphin actions are mediated by a cascade involving the spinal release of excitatory amino acids and prostaglandins. Unanesthetized rats with lumbar intrathecal injection and loop dialysis probes received intrathecal NMDA, dynorphin $A_{(1–17)}$, or dynorphin $A_{(2–17)}$. These agents elicited an acute release of glutamate, aspartate, and taurine but not serine. The dynorphin peptides and NMDA also elicited a long-lasting spinal release of prostaglandin E_2. Prostaglandin release evoked by dynorphin $A_{(2–17)}$ or NMDA was blocked by the NMDA antagonist amino-5-phosphonovalerate as well the cyclooxygenase (COX) inhibitor ibuprofen. To identify the COX isozyme contributing to this release, SC 58236, a COX-2 inhibitor, was given and found to reduce prostaglandin E_2 release evoked by either agent. Unexpectedly, the COX-1 inhibitor SC 58560 also reduced dynorphin $A_{(2–17)}$-induced, but not NMDA-induced, release of prostaglandin E_2. These findings reveal a novel mechanism by which elevated levels of spinal dynorphin seen in pathological conditions may produce hyperalgesia through the release of excitatory amino acids and in part by the activation of a constitutive spinal COX-1 and -2 cascade.

Key words: **dynorphin; NMDA; aspartate; glutamate; prostaglandin E_2; cyclooxygenase**

Introduction

Converging evidence suggests that hyperalgesia secondary to tissue or nerve injury and chronic opiate exposure is mediated through endogenous spinal dynorphin. Thus, peripheral inflammation increases dorsal horn prodynorphin mRNA (Ruda et al., 1988; Draisci et al., 1991; Dubner and Ruda, 1992), dynorphin content (Ruda et al., 1988; Dubner and Ruda, 1992; Riley et al., 1996), and extracellular spinal dynorphin (Riley et al., 1996; Pohl et al., 1997). Similarly, peripheral nerve injuries (Kajander et al., 1990; Draisci et al., 1991; Dubner and Ruda, 1992), including sciatic nerve ligation (Kajander et al., 1990; Malan et al., 2000) or cryoneurolysis (Wagner et al., 1993; Wagner and Deleo, 1996), increase spinal dynorphin content. Finally, chronic opioid treatments leading to hyperalgesia are associated with increased spinal dynorphin (Vanderah et al., 2000, 2001; Gardell et al., 2002). The relevance of these increases in spinal dynorphin is suggested by the antihyperalgesic action of intrathecal dynorphin antisera in models of tissue/nerve injury (Wagner and Deleo, 1996; Malan et al., 2000; Wang et al., 2001) and persistent opioid exposure (Vanderah et al., 2000; Vanderah et al., 2001). Moreover, mice with a deletion of the gene coding prodynorphin develop only transient hyperalgesia after nerve ligation (Wang et al., 2001).

Although these studies suggest that increased spinal dynorphin is a common element in hyperalgesia, the mechanism is unknown. Spinal dynorphin produces hyperalgesia (Vanderah et al., 1996). Because its actions are not reversed by naloxone and are produced by the des-tyrosyl form (dynorphin 2–17), the effect is nonopioid. Importantly, dynorphin hyperalgesia (Vanderah et al., 1996) is prevented by spinal NMDA receptor antagonism, like the hyperalgesia in several behavioral models (Trujillo and Akil, 1991; Tiseo and Inturrisi, 1993; Mao et al., 1994; Wagner and Deleo, 1996; Chaplan et al., 1997; Wang et al., 2001). Whether dynorphin can directly activate the NMDA receptor is not clear. Nevertheless, intrathecal dynorphin can evoke spinal glutamate release (Skilling et al., 1992). Such observations link spinal dynorphin to nociception, because intrathecal NMDA also produces hyperalgesia (Malmberg and Yaksh, 1992) and NMDA block diminishes hyperalgesia. Importantly, hyperalgesia produced by intrathecal NMDA is blocked by cyclooxygenase (COX) inhibitors (Malmberg and Yaksh, 1992), a finding consistent with the ability of intrathecal NMDA to evoke spinal prostaglandin E_2 (PGE_2) release (Malmberg and Yaksh, 1992; Sorkin, 1993; Dirig and Yaksh, 1999) through a constitutive spinal COX. The two COX isozymes (COX-1 and COX-2) are constitutively expressed in spinal cord (Svensson and Yaksh, 2002). The hyper-

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Correspondence should be addressed to Dr. Tony L. Yaksh, Anesthesiology Research Laboratory 0818, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0818. E-mail: tyaksh@ucsd.edu.

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algesia evoked by intrathecal NMDA is reversed by spinal COX-2, but not by COX-1, inhibition (Dirig et al., 1998), a finding consistent with block of spinal prostaglandin release by COX-2, but not COX-1, inhibition (Yaksh et al., 2001). These observations suggest that spinal dynorphin initiates a spinal release of excitatory amino acids and PGE_2. These studies confirm this hypothesis but further provide the novel finding that, unlike NMDA, spinal dynorphin-evoked PGE_2 release displays a COX-1 component.

Table 1. Baseline analyte concentrations in picomoles (amino acids) or femtomoles (PGE_2) per 10 min (mean ± SD)

	Aspartate	Glutamate	Serine	Taurine	PGE_2
Estimate	42.3 ± 14.2	187.6 ± 84.7	816.2 ± 252.4	763.0 ± 364.9	401.6 ± 261.3
Number	35	35	35	35	63
F ratio	1.57[a]	0.91[a]	3.31[a]	0.91[a]	2.04[b]
p value	0.217	0.239	<0.05	0.446	<0.05

[a]One-way ANOVA across treatment groups with 3,31 df.

[b]One-way ANOVA across treatment groups with 11,51 df.

Materials and Methods

Surgical preparation. Rats were anesthetized in a flow-through chamber saturated with isoflurane (5% in air; Abbott Laboratories, North Chicago, IL); after induction, isoflurane (2–3% in air) was delivered through a nose cone. After shaving and cleansing the scalp, the rat was placed in a stereotaxic head holder. Sharp dissection was used to separate muscles from the occipital bone and clear the atlanto-occipital membrane. A stab blade was used to make an incision in the dura mater perpendicular to the axis of the body, and the dialysis probe was inserted and advanced 9 cm caudad. The intrathecal portion of the probe consisted of a tubular 4 cm cellulose dialysis fiber (Filtral AN69HF; Cobe Laboratories, Denver, CO) bent double and connected by its ends to 7 cm of triple lumen polyethylene tubing (Spectranetics, Colorado Springs, CO). The dialysis fiber was connected to the outer lumens of the polyethylene tubing via 1 cm lengths of fused silica tubing (Polymicro Technologies, Phoenix, AZ) used as stents, with methacrylate adhesive (LocTite North America, Rocky Hill, CT) on the outside. Fine gauge wire (A–M Systems, Carlsborg, WA) lengthwise inside the dialysis fiber prevented crimping. The center lumen of the polyethylene tubing was used as an injection line; all three lumens were heat fused to short lengths of single lumen polyethylene tubing (PE-10; Clay Adams, Sparks, MD) to permit connection. This tubing was tunneled through the subcutaneous space, externalized over the frontal bones, and plugged with short lengths of wire (V. Mueller, McGaw Park, IL). After the incision was closed, fluids were administered [including loading the dialysis loop with penicillin–streptomycin solution (Invitrogen, Carlsbad, CA) and the infusion line with normal saline], and rats were placed in a warm chamber to recover consciousness. Afterward, rats were returned to the vivarium in their cages.

Microdialysis. Dialysis experiments were conducted after 2 or 3 d recovery from surgery. Previous work suggested that after this recovery period, consistent concentrations of analytes could be detected (Malmberg and Yaksh, 1995). The dialysis inflow and outflow tubing was connected to a two-channel fluid swivel (Eicom, Kyoto, Japan; Instech Laboratories, Plymouth Meeting, PA; Bioanalytical Systems, West Lafayette, IN). The effluent was routed to a programmable, refrigerated fraction collector (Eicom), while the infusate was delivered by syringe drivers at 10 μl/min (Harvard Apparatus, South Natick, MA). Artificial CSF was prepared such that the final composition was 151 mM sodium, 2.6 mM potassium, 132 mM chloride, 1.3 mM calcium, 0.9 mM Mg, 2.5 mM HPO_4, and 21 mM HCO_3 (all salts; EM Science, Gibbstown, NJ). The solution was bubbled with 5% CO_2 in air until clarity and filtered for sterility. Fraction lengths were either 10 min (excitatory amino acid experiments) or 20 min (PGE_2 experiments); fractions were collected at 4°C and frozen immediately (−20°C for amino acid analysis; −70°C for PGE_2 analysis). Initial experiments and previous work indicated that analyte levels reach steady state within 30 min of beginning perfusion (Malmberg and Yaksh, 1995). Data are expressed as percentages of the means of two to three baseline fractions. Intrathecal injections were given in a volume of 10 μl with a 10 μl saline flush, via the center lumen.

Amino acid analysis. Samples were thawed, and internal standard (400 pmol of methionine sulfone; Sigma, St. Louis, MO) was added immediately. The samples were dried under vacuum, then reconstituted in methanol–aqueous sodium acetate–triethylamine (all solvents and HPLC reagents; Fisher Chemical, Fair Lawn, NJ) and dried again. Amines were converted to chromogenic derivatives by conjugation with phenylisothiocyanate (5′ at room temperature in methanol–water–triethylamine). The reaction mixture was dried under vacuum and reconstituted in acetonitrile–aqueous sodium phosphate. Thirty microliter samples of this mixture were injected for HPLC using a Waters 712 autosampler. Separation of analyte peaks was achieved using a C18 column (MetaChem Polaris; 5 μm × 25 cm; MetaChem Technologies, Torrance, CA), maintained at 46°C, and gradient elution with a gradient cleanup step (60′; Waters 510 pump; 1 ml/min), controlled by computer. Peaks were detected by monitoring absorbance at 254 nm (0.05 absorbance units for full scale deflection; two samples per second; Agilent 1100 detector; Agilent Technologies, Palo Alto, CA). Ratios of analyte peak area to internal standard peak area were calculated for all samples; analyte mass was estimated by comparison to a 400 pmol standard solution. Estimates of aspartate, glutamate, serine, and taurine concentrations were obtained from the same chromatographic separation. Samples containing high dynorphin concentrations (i.e., when dynorphin was delivered in the dialysate) frequently had depressed internal standard peaks. Even under carefully controlled conditions, aspartate and glutamate concentrations could not be estimated correctly (data not shown). Therefore, samples were not included in the analysis if the internal standard peak area was <50% of its area in calibration standards. The limit of detection for each amino acid was typically 5 pmol in a sample.

PGE_2 analysis. PGE_2 concentrations were assayed using a commercially available kit (Assay Designs 90001; Assay Designs, Ann Arbor, MI). This kit uses a monoclonal antibody to PGE_2 (anchored to the plate via anti-IgG antibody) and PGE_2–alkaline phosphatase conjugate. The PGE_2 antibody has 70% cross-reactivity with PGE_1 and 16% cross-reactivity with PGE_3; no other cross-reactivities exceeding 2% are known (manufacturer's specifications). After incubation of the reagents and sample in the ELISA plate, activity bound to the solid phase is measured with paranitrophenylphosphate as absorbance at 450 nm. The amount of analyte in the sample is inferred from displacement of the PGE_2–alkaline phosphatase conjugate, and sample values are determined by reference to a standard curve. The linear detection range is generally 40–5000 pg/ml sample. Some rats were excluded because of elevated baseline PGE_2 concentrations (>1000 fmol/100 μl fraction). Control experiments indicated that dynorphin $A_{(2–17)}$ up to 10^{-5} M did not interfere with PGE_2 analysis (data not shown).

Data analysis. For each animal, the analyte levels measured before injection were averaged and used as the basis for calculating that animal's post-injection percentage of control values. Baseline values are presented in terms of analyte detected in one fraction of perfusate; for this analysis, flow through the dialysis circuit was assumed to be 10 μl/min. Amino acid data were analyzed by two-way repeated measures ANOVA; *post hoc* analyses used Fisher's PLSD test. Because PGE_2 values responded to treatment with a slower onset and offset, the response observed was integrated over time before analysis. This analysis consisted of one-way or two-way ANOVA.

Results

Basal release

Dialysate was collected from rats for 20 min before any injections to obtain basal release values for glutamate, aspartate, serine, and taurine. Pooled basal release values for these amino acids are summarized in Table 1. No significant difference in basal concentrations of glutamate, aspartate, and taurine were observed when compared among treatments. Serine exhibited significant ($p \leq 0.05$) variations in basal concentrations among the treatments,

Table 2. Amino acid release in percentages of baseline, 24 hr after injection

	Aspartate	Glutamate	Serine	Taurine
Artificial CSF ($n = 3–6$ per fraction)	85.5 ± 22.9%	82.6 ± 28.4%	82.3 ± 19.6%	85.2 ± 33.9%
Dynorphin $A_{(1–17)}$, 64 μg ($n = 7–8$ per fraction)	80.3 ± 18.4%	90.3 ± 21.1%	81.1 ± 21.1%	81.0 ± 21.5%
NMDA, 0.3 μg ($n = 4–5$ per fraction)	80.3 ± 18.4%	73.1 ± 31.3%	73.9 ± 31.4%	95.4 ± 54.7%

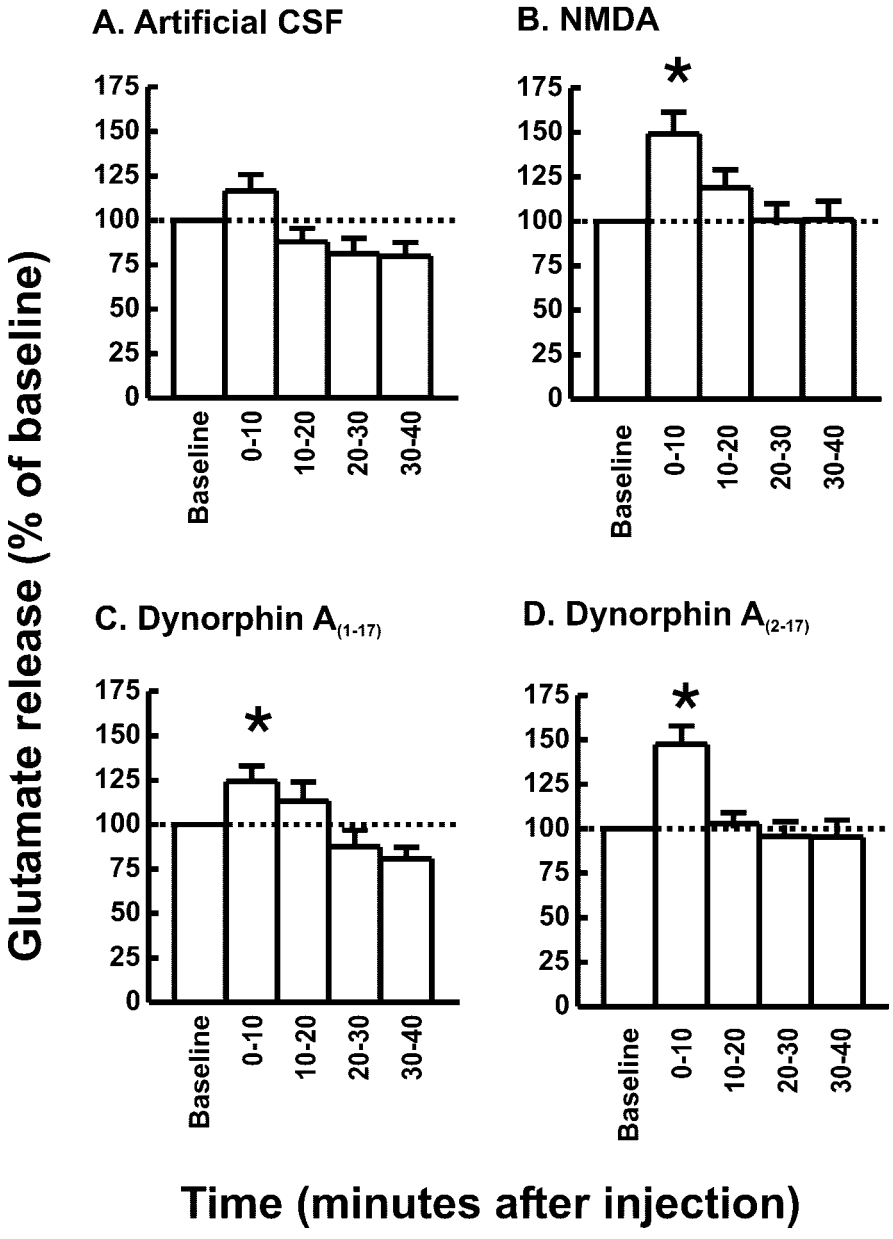

Figure 1. Evoked release of glutamate. The concentration of glutamate in dialysate was determined from samples collected at 10 min intervals for a total of 40 min. Baseline levels and glutamate levels at 10 min intervals after the intrathecal injection of artificial CSF (*A*), NMDA (4 nmol; *B*), dynorphin $A_{(1–17)}$ (30 nmol; *C*), or dynorphin $A_{(2–17)}$ (30 nmol; *D*) were measured. Data are expressed as percentages of the fractions collected before injection. Release after NMDA or des-tyrosyl dynorphin (but not native dynorphin) is different from artificial CSF (all $p < 0.05$).

which were attributable to a modest, yet significant, decrease in concentration observed in a single treatment group. Dialysate was also collected for at least 40 min before any injections to obtain basal concentrations of PGE_2. These values are also presented in Table 1. The data for evoked release were normalized and are presented as a percentage of the basal value for each group.

On the day after the evoked release experiments, groups of animals were reconnected to the dialysis apparatus. After a 30 min washout period, three dialysate fractions (10 min each) were collected and analyzed. Data are available on five animals treated with NMDA, eight treated with dynorphin $A_{(1–17)}$, and six treated with artificial CSF. When the three fractions are normalized to the pre-injection baseline of the previous day and averaged, the results show amino acid concentrations close to baseline (Table 2).

Evoked release of amino acids

The intrathecal injection of NMDA (4 nmol), dynorphin $A_{(1–17)}$, or dynorphin $A_{(2–17)}$ (30 nmol) each produced a significant ($p \leq 0.05$), but short-lived, increase in the release of glutamate (Fig. 1). The significantly elevated concentrations of glutamate were detected only in the fractions collected during the first 10 min of dialysis. Glutamate release was increased to 149 ± 12%, 124 ± 9%, and 147 ± 10% by NMDA, dynorphin $A_{(1–17)}$, and dynorphin $A_{(2–17)}$, respectively. Similarly, aspartate and taurine were significantly ($p \leq 0.05$) elevated during the initial 10 min of dialysis. The corresponding values for aspartate were 166 ± 23%, 115 ± 16%, and 139 ± 10%, and those for taurine were 141 ± 11%, 112 ± 6%, and 146 ± 8%. In contrast, serine concentrations did not change significantly with any treatment and ranged from 101 ± 4% to 113 ± 6%. This failure to see a change in serine provides support that the elevated release of the other three amino acids was not secondary to a general increase in the concentrations of all analytes. The intrathecal injection of artificial CSF did not produce any changes in release of amino acids.

Evoked release of PGE_2

The intrathecal injection of NMDA (4 nmol), dynorphin $A_{(1–17)}$, or dynorphin $A_{(2–17)}$ (30 nmol) each evoked the release of PGE_2, as demonstrated by a significant ($p \leq 0.05$) increase in the concentration of PGE_2 in the dialysate (Fig. 2). In contrast to the evoked release of the amino acids, the release of PGE_2 demonstrated a slower time course. The concentrations of PGE_2 in response to intrathecal injections of NMDA or of the dynorphins rose slowly and were maximal in the fractions collected between 40 and 60 min after the injection (Fig. 2). The concentration of PGE_2 was increased to 611 ± 174%, 469 ± 169%, and 530 ± 106% by NMDA, dynorphin $A_{(1–17)}$, and dynorphin $A_{(2–17)}$, respectively. The intrathecal injection of artificial CSF did not produce any changes in concentrations of PGE_2 in the dialysate (Fig. 2).

Antagonism of NMDA-evoked release of PGE_2

The intrathecal injection of the NMDA antagonist AP-5 (30 μg) before the intrathecal injection of NMDA completely blocked the NMDA-evoked release of PGE_2 (Fig. 3*A*). The concentration of PGE_2 in the dialysate collected from 40 to 60 min after NMDA injection was 52% of the baseline value. Likewise, the injection of ibuprofen (30 mg/kg, i.p.) blocked NMDA-evoked release of PGE_2 (Fig. 3*B*). The detected amount of PGE_2 in dialysate collected 40–60 min after NMDA was 42 ± 14% of the baseline value, indicating a substantial decrease in PGE_2 release. In contrast, the injection of 30 mg/kg (intraperitoneal) of the selective COX-1 inhibitor SC 58560 (Mazario et al., 2001) did not alter NMDA-evoked release of PGE_2 (Fig. 3*C*). The maximal evoked release occurred in the fraction collected 40–60 min after NMDA and was 587 ± 267% of the baseline value and was not significantly different ($p > 0.05$) from that evoked by NMDA alone (Fig. 2). As with ibuprofen, the intraperitoneal injection of 30 mg/kg of the selective COX-2 inhibitor SC 58236 (Govoni et al., 2001) completely blocked the NMDA-evoked release of PGE_2 (Fig. 3*D*). The concentration of PGE_2 detected 40–60 min after NMDA was 12 ± 2% of the baseline value, indicating a substantial decrease in NMDA-evoked PGE_2 release. ANOVA, followed by Fisher's PLSD test revealed that the decreases in NMDA-evoked release of PGE_2, compared with NMDA alone, were significant in the presence of AP-5 ($p < 0.05$), ibuprofen ($p < 0.005$), and SC 58236 ($p < 0.005$).

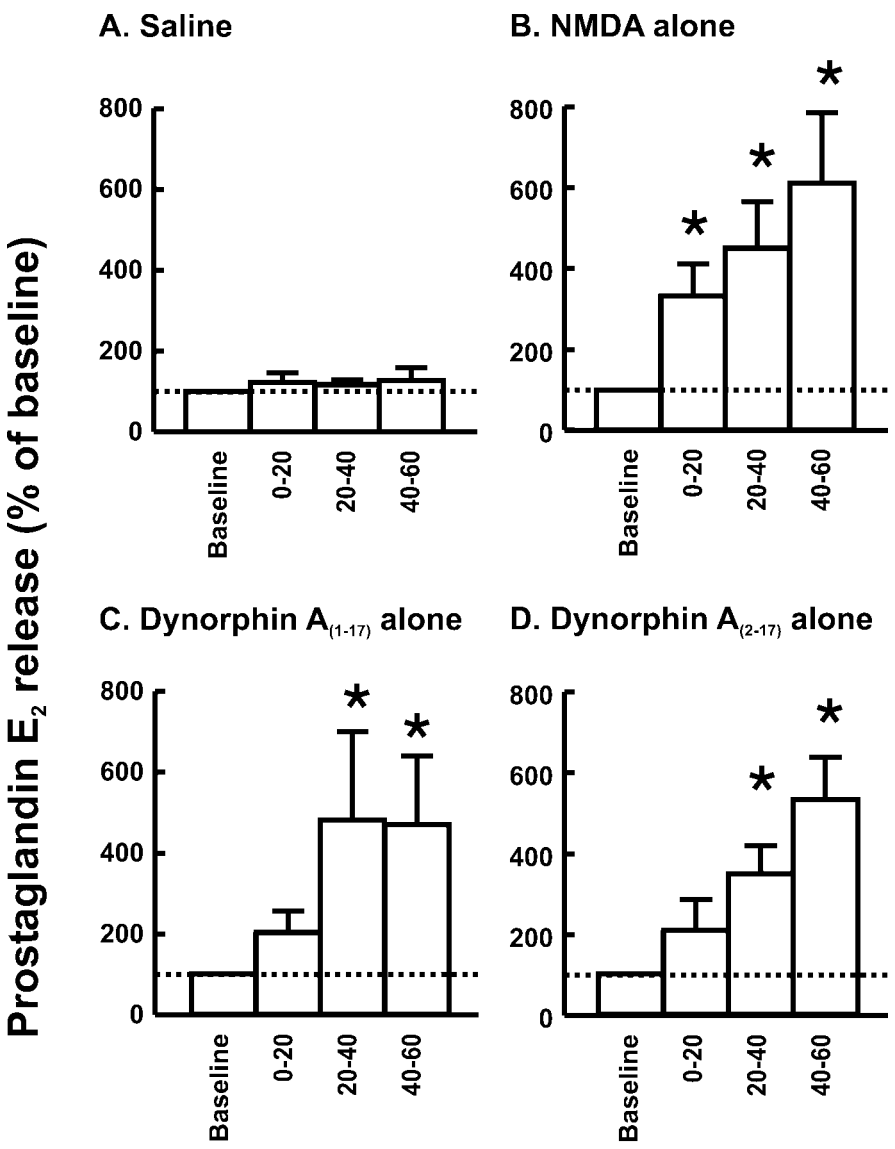

Figure 2. Evoked release of PGE_2. The concentration of PGE_2 in dialysate was determined from samples collected at 20 min intervals for a total of 60 min. Baseline levels of PGE_2 and PGE_2 levels at 20 min intervals after the intrathecal injection of artificial CSF (*A*), NMDA (4 nmol; *B*), dynorphin $A_{(1-17)}$ (30 nmol; *C*), or dynorphin $A_{(2-17)}$ (30 nmol; *D*) were measured. Data are expressed as percentages of the fractions collected before injection. Release after NMDA (but not dynorphin treatment) is not significantly different from that seen after injection of artificial CSF (all $p > 0.05$).

Antagonism of dynorphin $A_{(2–17)}$-evoked release of PGE_2

The intrathecal injection of the NMDA antagonist AP-5 (30 μg) before the intrathecal injection of dynorphin $A_{(2–17)}$ substantially blocked the release of PGE_2 evoked by dynorphin $A_{(2–17)}$ (Fig. 4*A*). The concentration of PGE_2 in the dialysate collected between 40 and 60 min after the intrathecal administration of dynorphin $A_{(2–17)}$ was 140 ± 28% of the baseline value. Similarly, the injection of ibuprofen (30 mg/kg, i.p.) significantly ($p \leq 0.01$) reduced the dynorphin $A_{(2–17)}$-evoked release of PGE_2 when compared with dynorphin $A_{(2–17)}$ alone (Fig. 4*B*). The detected amount of PGE_2 in dialysate collected 40–60 min after dynorphin $A_{(2–17)}$ was 275 ± 68% of the baseline value. The intraperitoneal injection of 30 mg/kg of the selective COX-1 inhibitor SC 58560 produced a similar reduction in the release of PGE_2 evoked by dynorphin $A_{(2–17)}$ (Fig. 4C). The maximal evoked release occurred in the fraction collected 40–60 min after dynorphin $A_{(2–17)}$ was 239 ± 20% of the baseline value and was significantly different ($p \leq 0.05$) from that evoked by dynorphin $A_{(2–17)}$ alone (Fig. 2). The intraperitoneal injection of 30 mg/kg of the selective COX-2 inhibitor SC 58236 completely blocked the dynorphin $A_{(2–17)}$-evoked release of PGE_2 (Fig. 4*D*). The concentration of PGE_2 detected 40–60 min after dynorphin $A_{(2–17)}$ was 5.2 ± 0.2% of the baseline value, indicating a substantial decrease in dynorphin $A_{(2–17)}$-evoked PGE_2 release.

Discussion

Hyperalgesia attributable to tissue and nerve injury or chronic opiate exposure is associated with increased spinal dynorphin expression (Ruda et al., 1988; Kajander et al., 1990; Dubner and Ruda, 1992; Pohl et al., 1997; Malan et al., 2000). Intrathecal dynorphin induces hyperalgesia, whereas manipulations interfering with dynorphin signaling block dynorphin-associated hyperpathia (Malan et al., 2000; Vanderah et al., 2000; Burgess et al., 2002; Gardell et al., 2002). Here, we show that intrathecal dynorphin initiates glutamate release and a delayed PGE_2 release of extended duration. The ability of dynorphin to release glutamate

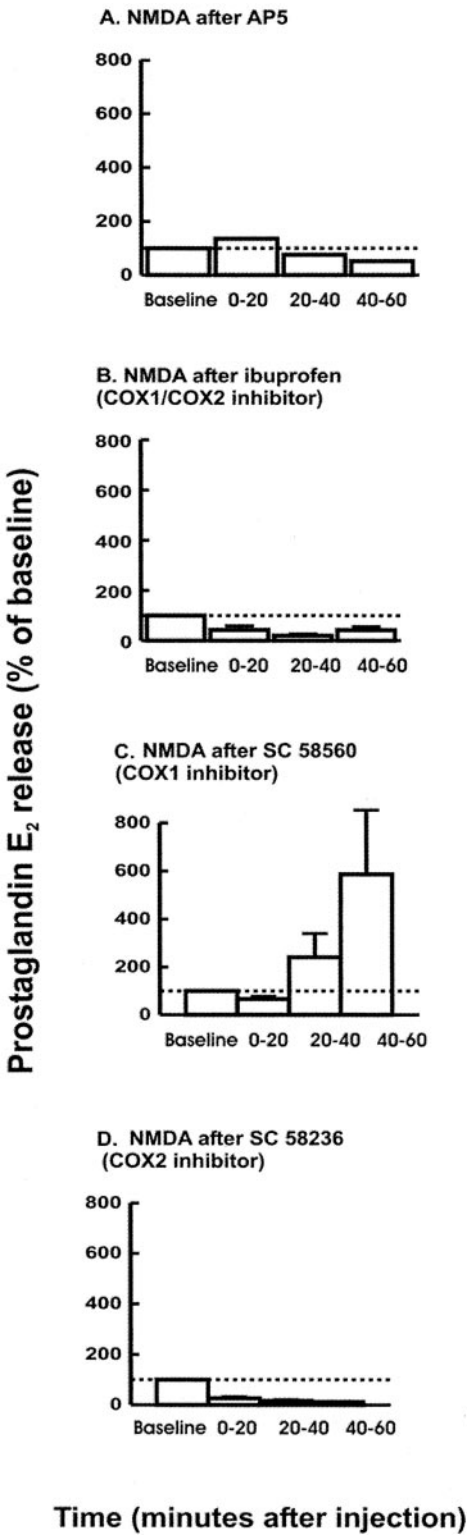

Figure 3. NMDA-evoked release of PGE_2. Dialysate PGE_2 concentrations were monitored before and after intrathecal injection of NMDA (4 nmol). Samples were collected before injection and at 20 min intervals after the intrathecal injection of NMDA. The rats were pretreated with AP-5 (30 μg, intrathecal) 10 min before NMDA (*A*), ibuprofen (30 mg/kg, i.p.) 50 min before NMDA (*B*), the COX-1 inhibitor SC 58560 (30 mg/kg, i.p.) 30 min before NMDA (*C*), or the COX-2 inhibitor SC 58236 (30 mg/kg, i.p.) 30 min before NMDA (*D*). The pretreatment with AP-5, ibuprofen, or the COX-2 inhibitor SC 58236 significantly ($p \leq 0.05$) reduced NMDA-evoked release of PGE_2. In contrast, the pretreatment with the COX-1 inhibitor SC 58560 did not reduce NMDA-evoked release of PGE_2.

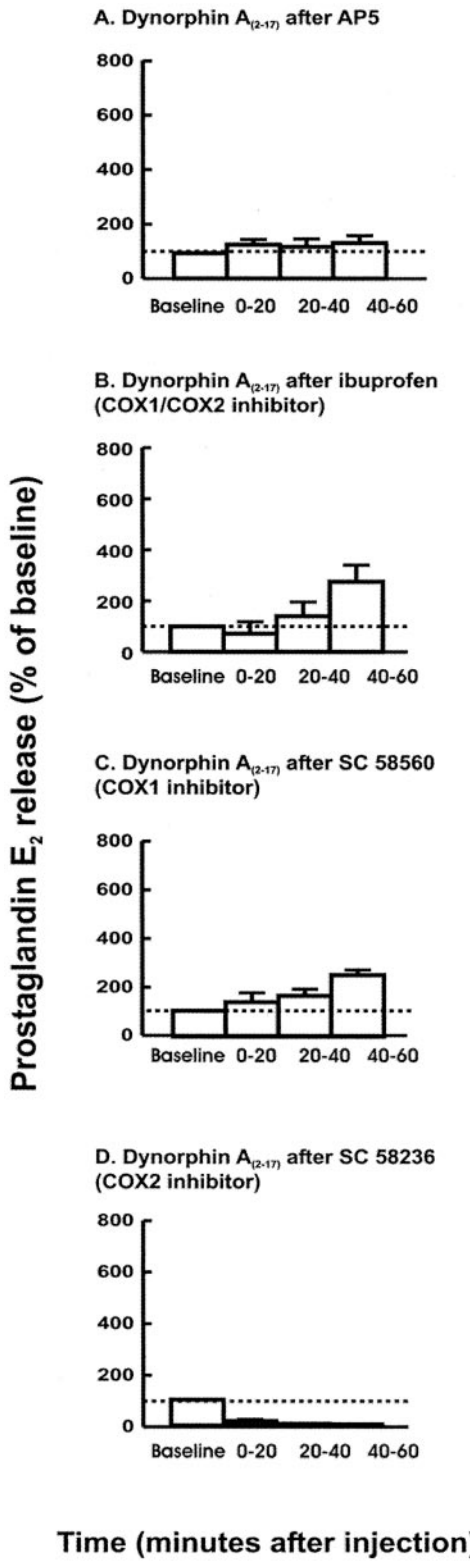

Figure 4. Dynorphin $A_{(2-17)}$-evoked release of PGE_2. The dialysate PGE_2 concentrations were monitored before and after intrathecal injection of dynorphin $A_{(2-17)}$ (30 nmol). Samples were collected before injection and at 20 min intervals after the intrathecal injection of dynorphin. The rats were pretreated with AP-5 (30 μg, intrathecal) 10 min before dynorphin (*A*), ibuprofen (30 mg/kg, i.p.) 50 min before dynorphin (*B*), the COX-1 inhibitor SC 58560 (30 mg/kg, i.p.) 30 min before dynorphin (*C*), or the COX-2 inhibitor SC 58236 (30 mg/kg, i.p.) 30 min before dynorphin (*D*). The pretreatment with AP-5, ibuprofen, the COX-1 inhibitor SC 58560, or the COX-2 inhibitor SC 58236 all significantly (*$p \leq 0.05$) reduced dynorphin $A_{(2-17)}$-evoked release of PGE_2.

and PGE_2 through COX-2 suggests a cascade similar to that reported for tissue injury (Yaksh et al., 1999). Unexpectedly, we found that dynorphin-evoked PGE_2 release, unlike that evoked by NMDA, has an additional requirement for COX-1 activity.

Membrane action of dynorphin

In the present study, both dynorphin $A_{(1–17)}$ and dynorphin $A_{(2–17)}$ stimulated amino acid and PGE_2 release. Binding studies show significant affinity of dynorphin for the three opiate receptors (Quirion and Pert, 1981), as well as ORL-1 (Dooley and Houghten, 2000). In contrast, dynorphin $A_{(2–17)}$ does not bind to either opiate (Chavkin and Goldstein, 1981) or to ORL-1 (Meng et al., 1996) receptors, emphasizing the opioid independence of the stimulatory action. Given the rate of dynorphin metabolism (Young et al., 1987), the des-tyrosyl products are likely to be a reliable component of the effects of either exogenous or endogenous dynorphin. Although not systematically examined, dynorphin $A_{(1–17)}$ seemed less effective than dynorphin $A_{(2–17)}$ at stimulating release. This previously reported observation (Hauser et al., 1999) may reflect an antagonism between possible opiate and nonopiate effects.

The mechanism by which dynorphin $A_{(2–17)}$ acts is not understood (Vanderah et al., 2000; Burgess et al., 2002; Gardell et al., 2002). The ability of competitive (AP-5) and channel blocking (MK801) antagonists to block dynorphin-stimulated glutamate (Faden, 1992; Skilling et al., 1992) and PGE_2 release (present study) indicate that dynorphin acts in part through an NMDA receptor. Several possible interactions between dynorphin and the NMDA receptor may occur. Dynorphin is an NMDA receptor ligand (Massardier and Hunt, 1989; Tang et al., 1999). Both antagonist (Shukla et al., 1997; Tang et al., 1999) and agonist effects (Lai et al., 1998) have been described. Alternately, NMDA-mediated effects of dynorphin can represent an indirect interaction mediated by the initial release of excitatory amino acids that serve than to stimulate NMDA receptors. A nonopiate, non-NMDA site for dynorphin binding has been proposed (Tang et al., 2000).

Amino acid release

Aspartate, glutamate, and taurine (but not serine) displayed a transient increase immediately after injection of dynorphin $A_{(1–17)}$/dynorphin $A_{(2–17)}$ or NMDA. The evoked increase in glutamate, aspartate, and taurine release are similar to that noted after somatic stimuli [e.g., intraplantar formalin (Malmberg and Yaksh, 1995) and spinal opiate withdrawal (Jhamandas et al., 1996)], leading to hyperalgesia. These results mirror those reported previously in spinal cord and hippocampus (Faden, 1992; Skilling et al., 1992). Although its role is not understood, spinal taurine release occurs in concert with excitatory amino acids after intraplantar formalin (Malmberg and Yaksh, 1995) and opiate withdrawal (Jhamandas et al., 1996). Taurine release is considered to be a marker for activation and increased intracellular calcium (Foos and Wu, 2002). Serine is not considered to be a regulated neurotransmitter, and its modest change in contrast to those observed for excitatory amino acids and PGE_2 serves as a marker for the absence of nonspecific changes in extracellular content or dialysis probe function.

Prostaglandin release

Intrathecal injection of dynorphin $A_{(1–17)}$/dynorphin $A_{(2–17)}$ or NMDA resulted in a delayed increase in extracellular PGE_2 concentrations. This increase by NMDA and dynorphin was reduced by an NMDA antagonist, emphasizing the importance of NMDA receptor activation. These results are consistent with our work showing that intrathecal NMDA stimulates PGE_2 release (Malmberg and Yaksh, 1995; Dirig and Yaksh, 1999) and suggest that dynorphin-evoked PGE_2 release occurred by direct activation of the NMDA receptor or through an initial release of excitatory amino acids as considered above. The apparent difference in sensitivity of the PGE_2 release evoked between NMDA and dynorphin to the COX-1 inhibitor SC 58560 (see below) now suggests an additional action of dynorphin, independent of NMDA receptor activation.

Spinal COX isozymes

The immediate suppression of NMDA and dynorphin effects by ibuprofen suggests that spinal release of PGE_2 requires constitutive COX activity and confirms that the prostanoids arise from a COX-dependent pathway. There are at least two COX isozymes, COX-1 and –2, expressed constitutively in spinal cells (Svensson and Yaksh, 2002). The substantial effect of SC 58236 on both NMDA- and dynorphin-evoked PGE_2 release implicates COX-2 in both effects. In contrast, SC 58560 had no effect on the prostaglandin release initiated by NMDA but suppressed that evoked by dynorphin. This argues that dynorphin exerts an action in addition to one mediated though NMDA receptor COX-2 linkage.

Dynorphin, NMDA, and the spinal COX cascade

Previous work shows that tissue injury/inflammation, nerve injury, or spinal opiate withdrawal can initiate hyperalgesia. Many of these models share an important role for the spinal release of glutamate, as evidenced by (1) reversal of hyperalgesia by spinal NMDA antagonists (Ren et al., 1992; Mao et al., 1994; Chaplan et al., 1997) and (2) increased spinal glutamate release (Malmberg and Yaksh, 1995; Jhamandas et al., 1996). These observations are consistent with the hyperalgesia produced by intrathecal NMDA (Malmberg and Yaksh, 1992). Current evidence suggests that spinal NMDA receptors initiate a cascade involving the release of prostaglandins that, through prostanoid receptors, facilitates the spinal response to afferent stimuli (Svensson and Yaksh, 2002). Thus, after intrathecal NMDA or substance P (Malmberg and Yaksh, 1992), paw inflammation (Dirig et al., 1998), nerve injury (Ma et al., 2002; Hefferan et al., 2003; Ma et al., 2003), or opiate withdrawal (Trang et al., 2002), spinal COX inhibitors diminish hyperpathia. These observations are consistent with an increase in spinal PGE_2 release produced by these treatments (Malmberg and Yaksh, 1995; Jhamandas et al., 1996; Tegeder et al., 2001; Yaksh et al., 2001). Because prostanoids enhance afferent terminal release of peptides (Vasko, 1995), the dynorphin-evoked prostaglandin release provides a mechanism for dynorphin-induced release of afferent peptides (Skilling et al., 1992; Arcaya et al., 1999; Gardell et al., 2002). Whether the dynorphin effect on primary afferent release is sensitive to COX-1 or -2 inhibition is not known.

Investigation of the COX isozyme responsible for these effects suggests an important role for spinal COX-2 and not for COX-1 after intrathecal substance P (Yaksh et al., 2001), NMDA (as shown in the present study), or paw inflammation (Dirig et al., 1998). In contrast, after nerve injury, both COX-1 and COX-2 inhibitors have been reported effective. This suggests that nerve injury allodynia, in contrast to inflammatory pain or the effects of spinal NMDA or NK1 receptor activation, is mediated by the activity of both COX enzymes (Parris et al., 1996; Ma et al., 2002; Hefferan et al., 2003). A multiplicative interaction between spinally administered inhibitors of COX-1 and COX-2 against neu-

ropathic pain has been reported (Lashbrook et al., 1999), suggesting a joint role of these enzymes in some, but not all, spinal facilitated states. The increase in dynorphin expression after such nerve injury, thus, provides an important corollary to these observations on the role for both COX enzymes after intrathecal dynorphin.

This role of COX-1 in addition to COX-2 distinguishes the spinal effects of dynorphin from those of NMDA. Were spinal dynorphin to be acting through the release of glutamate or on the NMDA receptor, its downstream COX enzyme pharmacology would resemble that of NMDA. Accordingly, we now suggest that spinal dynorphin acts through an NMDA receptor-dependent mechanism (as suggested by the antagonist results) as well as through an NMDA-independent component that leads to a COX-1 and COX-2 cascade. Accordingly, our organizing hypothesis is that an index of an NMDA contribution to dynorphin action will be a COX-2, but not COX-1, inhibitor sensitivity. Conversely, the NMDA-independent action will include a COX-1 contribution.

These studies provide novel insight into spinal mechanisms promoting hyperalgesia attributable to inflammation or nerve injury. The increased production of spinal dynorphin that occurs in response to injury may enhance neurotransmitter release and produce a state of hyperalgesia through multiple COX cascades. The present findings also provide a mechanism whereby NSAIDs, particularly COX-1 inhibitors, could work as therapeutic adjuncts for the treatment of neuropathic pain. The present study, thus, provides a basis for the role of spinal dynorphin in pathological conditions and the production of clinically important states of hyperalgesia.

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