Pharmacology of the Spinal Action of KETOROLAC, Morphine, ST-91, U50488H, and L-PIA on the Formalin Test and an Isobolographic Analysis of the NSAID Interaction

Annika B. Malmberg, M.S.,* Tony L. Yaksh, Ph.D.†

Background: Noxious cutaneous stimuli enhance spinal excitability. The behavioral correlate to this response is found in the rat formalin test, in which formalin injection into the hindpaw evokes signs of nociception (flinching and licking of the injected paw) with acute (phase 1) and delayed-hyperalgesic (phase 2) components.

Methods: The effect of intrathecal morphine (µ agonist), U50488H (a k agonist), ST-91 (an δ agonist), L-PIA (an adenosine A1 agonist), and ketorolac (a nonsteroidal antiinflammatory drug, or NSAID), were examined in rats undergoing the formalin test. Spinal interactions between ketorolac and the µ, k, δ, and adenosine A1 agonists were assessed using isobolographic analysis.

Results: Morphine and ST-91 caused a dose-dependent suppression of phase 1 and phase 2 of the formalin test, while U50488H and L-PIA had little effect on phase 1, but caused dose-dependent depression of phase 2. Intrathecal ketorolac inhibited the phase 2 response, but had limited effect on phase 1. The isobolographic analysis revealed a significant synergy (with fractional dose ratios of less than 1) between ketorolac and morphine or ST-91 for phase 1 and phase 2, but an additive interaction was found between ketorolac and L-PIA or U50488H.

Conclusions: These observations offer systematic support for the powerful interaction between NSAIDs and opioids and certain other analgesics in clinical pain states. These studies also demonstrate that spinal synergy is not a common property of all interactions. Thus, the NSAID synergy appears to occur with agents that exert a concurrent action both pre- and postsynaptic to the primary afferents. (Key words: Agonists, adenosine A1; L-PIA. Analgesics, nonsteroidal antiinflammatory drugs: ketorolac. Analgesics, µ opioid: U50488H. Analgesics, µ opioid: morphine. Spinal cord. Sympathetic nervous system, δ agonist: ST-91.)

AFTER an acute injury, repetitive C-fiber input evokes a state of spinal facilitation, referred to as "windup," in which sequential stimuli evoke progressively greater responses in dorsal horn wide dynamic range (WDR) neurons and an increase in the size of their respective receptive fields. In the rat, a small volume of dilute formalin injected into the paw of the rat produces an initial barrage of nerve activity (1–10 min), followed, over the next 50 min, by a low level of discharge in C-fiber afferents. Behaviorally, the formalin results in a biphasic paw-flinching behavior with a time course and amplitude that correspond to activity in spinal WDR neurons. The dissociation of the C-fiber activity from the intensity of behavior indicates that the second phase of the formalin response reflects hyperalgesia. The windup and hyperalgesic component of the second phase of the formalin test are mediated by substance P receptors of the neurokinin-1 (NK-1) type8 and glutamate receptors of the N-methyl-D-aspartate (NMDA) type.8-11 These spinal receptor systems exert their effects by activation of several intracellular processes, including the formation of prostaglandins. Spinal nonsteroidal antiinflammatory drugs (NSAIDs) reduce the behavioral hyperalgesia evoked by the spinal action of substance P and NMDA. However, although NK-1 and NMDA antagonists and NSAIDs are able to suppress the second phase of the formalin test, they have little de-

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Anesthesiology, V 79, No 2, Aug 1993
detectable effect on the acute afferent-evoked excitation that occurs during the phase 1 response of the formalin test.\textsuperscript{8,11,13} It is, therefore, possible to discern differences in the pharmacology of the acute pain response generated by an acute stimulus and the subsequent hyperalgesic component initiated by that stimulus.

Several classes of receptor-selective agents act spinally to alter nociceptive processing. \(\alpha_2\) and \(\mu\) opioid receptor agonists produce a powerful analgesia by a presynaptic inhibition of C-fiber neurotransmitter release,\textsuperscript{14-17} and a postsynaptic hyperpolarization of WDR neurons.\textsuperscript{18-20} Adenosine and \(\kappa\) receptor agonists depress firing of dorsal horn neurons, but neither diminishes release of spinal C-fiber transmitters,\textsuperscript{15,21,22} excluding a presynaptic action on primary afferents. Spinal \(\mu\) opioid receptor agonists have been shown to produce a dose-dependent depression of the phase 1 and 2 response,\textsuperscript{6,7,11,23} and \(\kappa\) opioid receptor agonists inhibit phase 2 formalin response.\textsuperscript{6,3} Characteristics of spinal \(\alpha_2\) and adenosine agonists on the formalin test are unknown.

The central action of NSAIDs, and the probable importance of a hyperalgesic component in postinjury pain states, may account, in part, for the potency of NSAIDs in diminishing postoperative pain in humans. Combinations of NSAIDs with opioids are used in clinical practice to lower postoperative opioid requirements.\textsuperscript{24-28} In cancer pain management, World Health Organization guidelines\textsuperscript{29} emphasize the concurrent use of NSAIDs to enhance opioid effects and decrease the opioid dose necessary to achieve satisfactory pain relief.\textsuperscript{30,31} In spite of the frequent use of these combinations, there are few systematic investigations of this purported interaction.

In the current study, we sought to: 1) define the effects of \(\mu\) opioid (morphine), \(\alpha_2\) adrenergic (ST-91), \(\kappa\) opioid (U50488H), and \(\Lambda_1\) adenosine (L-PtIA) receptor agonists on the phase 1 and phase 2 of the formalin response; 2) assess the antagonist pharmacology of the spinal action of these agents; and 3) characterize the spinal interaction between ketorolac, a cyclooxygenase inhibitor, and the several agonists, using isobolographic analysis.\textsuperscript{32,33}

**Materials and Methods**

**Animal Preparation**

Male Sprague-Dawley rats (280–320 g; Harlan Industries, Indianapolis, IN) were implanted with chronic lumbar intrathecal catheters under halothane (2%) anesthesia according to a modification of the method described by Yaksh and Rudy.\textsuperscript{34} Briefly, through an incision in the atlantoccipital membrane, a polyethylene (PE-10) catheter was advanced caudally, extending to the rostral edge of the lumbar enlargement. After implantation of intrathecal catheters, rats were housed in individual steel cages. Intrathecal injection studies were carried out 7–10 days after surgery. Only animals with normal motor function were used. Experiments were carried out according to a protocol approved by the Institutional Animal Care Committee of the University of California, San Diego.

**Drugs and Injection**

Drugs for intrathecal administration were mixed such that all doses were delivered in a total volume of 10 \(\mu\)l, followed by 10 \(\mu\)l saline to flush the catheter. The following drugs were used in this study: ketorolac (ketorolac tromethamine; MW = 376; Toradol intramuscularly, inj. syringe 30 mg/ml, Syntax, Palo Alto, CA), morphine (morphine sulfate; MW = 334; Merck, Sharpe and Dohme, West Point, PA), ST-91 (2-[2,6-diethylphenylamino]-2-imidazoline; \(MW = 253; \) Boehringer Ingelheim, Ltd., Ridgefield, CT), U50488H (trans[\(+\)-3,4-dichloro-N-methyl-N-[2-[1-pyrrolidinyl]cyclohexyl]-benzacetamide methane sulfonate salt; \(MW = 465; \) Upjohn, Kalamazoo, MI), L-PtIA (N\textsuperscript{6}'-[L-2-phenylisopropyl]-adenosine; \(MW = 385; \) Sigma, St. Louis, MO), naloxone-HCl (\(MW = 327; \) DuPont, Wilmington, DE), yohimbine (\(MW = 391; \) Sigma) nor-Binaltorphimine dihydrochloride (norBNI; \(MW = 735; \) Research Biochemicals, Natick, MA), and caffeine sodium benzoate (50:50 \(^{\text{w/s}}\) mixture; \(MW = 194; \) Sigma). Ketorolac, morphine, naloxone, ST-91, yohimbine, U50488H, and caffeine were further diluted in physiologic saline (0.9% \(^{\text{w/v}}\)). L-PtIA and norBNI were dissolved in 2-hydroxypropyl-\(\beta\)-cyclodextrin (cyclodextrin; Research Biochemicals) with a final concentration of 5%.

**Formalin Test**

In order to perform the formalin injection, the rats were anesthetized briefly with halothane (3%). When there was a momentary loss of spontaneous movement with preservation of the deep spontaneous respiration and blink and pinnae reflexes, 50 \(\mu\)l of 5% formalin solution was injected subcutaneously into the dorsal surface of the right hind paw with a 30-G needle. The rat was then placed, for observation, in an open plexiglas chamber with a mirror placed on the opposite side.
to allow for unhindered observation of the formalin-injected paw. Pain behavior was quantified by periodically counting the incidence of spontaneous flinching/shaking of the injected paw. The flinches were counted for 1-min periods at 1–2 and 5–6 min, and then at 5-min intervals during the interval from 10 to 60 min after formalin injection. Two phases of spontaneous flinching behavior were observed. Phase 1 started immediately after formalin injection and lasted through the second observation interval (5–6 min). Phase 2 began after 10 min, and maximum response was typically observed around 25–35 min after the formalin injection. For the purpose of data analysis, the second phase was further divided into two phases: phase 2A (10–39 min) and phase 2B (40–60 min).

After the observation period of 1 h, animals were immediately killed with an overdose of barbiturate mixture (Beuthanasia, 50 mg/kg, intraperitoneally; Schering-Plough, Animal Health, Kenilworth, NJ).

**Behavioral Testing**

The general behavior of all of the rats was carefully observed and tested. Motor function was examined by the placing/stepping reflex; the rat was gently grasped around the torso, and the dorsum of either hindpaw was drawn across the edge of a table. Normal ambulation and righting were assessed by placing the rat horizontally with its back on the table, which normally gives rise to an immediate, coordinated twisting of the body to an upright position. The presence of allodynia was examined by looking for agitation (escape or vocalization) evoked by lightly stroking the flank of the rat with a pencil.

**Experimental Paradigm**

Because of the large number of experimental groups employed in these studies, control animals (animals which received no injection, or intrathecal saline or 5% cyclodextrin) were evaluated sporadically during the course of the study period.

**Agonist and NSAID Studies.** The first series of experiments was performed to determine the dose-dependency and time course of the analgesic actions of intrathecally administered μ opioid, δ adrenergic, and κ and adenosine receptor agonists on the formalin response. Results from intrathecal injection of NSAIDs, including ketorolac, on the formalin response have been reported previously. Based on previous studies, the dose-response curves were carried out with intrathecal injection of the agonists 10 min before the formalin test. The highest dose examined in the dose-response studies was determined on the basis of three factors: 1) when the dose did not differ in effect by more than 10% of the maximum effect achieved by the preceding lower dose; 2) when a maximal effect was obtained (total suppression of the formalin response); or 3) when the dose resulted in adverse effects such as motor impairment. The dose defined by these criteria was termed the just-maximally effective dose (JME) of the agonist.

**Antagonist Studies.** Antagonists were administered at a time that, based on previous studies or preliminary data, corresponded to the time of peak effects for the agonist and antagonist. The Agonist doses employed were those that produced a just-maximal effect, as determined in the dose-response studies. Antagonist doses were based on previous work in the laboratory (opioid [naloxone], δ2 adrenergic [yohimbine], κ [norBNI], and adenosine [caffeine]). The doses and the time of treatment were as follows: naloxone, 2.8 μM/kg intraperitoneally 5 min after agonist injection; yohimbine, 76 nm intrathecally 30 min before the formalin test; norBNI, 20 nm intrathecally concurrent with agonist; and caffeine 2 μM intrathecally concurrent with agonist. The respective doses of antagonists were also given with saline as control.

**Interaction Studies.** To characterize the functional interaction between spinal NSAIDs and spinal antinociceptive receptor agonists, an isobolographic analysis of interactions was used. The method is based on comparisons of doses that are determined to be equieffective. From the dose-response curves of the several agents alone, the respective ED50 values (effective dose resulting in a 50% reduction of the control formalin response) are determined. Subsequently, a dose-response curve is obtained by coadministration of the two drugs in a constant dose-ratio based on the ED50 values of the single agents. The equieffective dose ratios were: 1:1.4 for morphine and ketorolac, 1:1.3 for ST-91 and ketorolac, 30:1 for U50488H and ketorolac, and 1:6.7 for L-PIA and ketorolac. From the dose-response curve of the combined drugs, the ED50 value of the total dose of the mixture was calculated, and, based on the known dose ratio, the single doses of the agents in the combination were obtained for plotting on the isobologram.

The isobolograms were constructed as described previously. Briefly, the ED50 values of the single agents

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‡ Malmberg AB: Unpublished observations.
were plotted on the X and Y axis, respectively. The theoretically additive dose combination was calculated according to the method described by Tallarida.\textsuperscript{35} From the variance of the total dose, individual variances for the agents in the mixture were obtained. For statistical comparison of the difference between the theoretical additive point and the experimentally derived \( ED_{50} \) value, Student's \( t \) test was used. An experimental \( ED_{50} \) that was significantly less than the theoretical additive \( ED_{50} \) (\( P < 0.05 \)) was considered to indicate a multiplicative (more than additive) interaction between the agents.

To describe the magnitude of the interaction, a total fraction value (see formula below) was calculated. The \( ED_{50} \) values were normalized, such that the \( ED_{50} \) value of an agent given alone (the value on the axis) was given the number 1. The fractional value describes the experimental \( ED_{50} \) as a fraction of the additive \( ED_{50} \). Values near 1 indicate additive interaction, values greater than 1 imply an antagonistic interaction, and values less than 1 indicate a synergistic, multiplicative interaction.

\[
\frac{\text{ED}_{50} \text{ dose in combination of drug 1}}{\text{ED}_{50} \text{ value for drug 1 given alone}} + \frac{\text{ED}_{50} \text{ dose in combination of drug 2}}{\text{ED}_{50} \text{ value for drug 2 given alone}}
\]

Antagonist Studies of Interaction. The combination that resulted in a maximal effect was used for antagonist studies. Naloxone (2.8 \( \mu \)g/kg) was given intraperitoneally 5 min before formalin and 5 min after the intrathecal coadministration of morphine (1.2 nm) and ketorolac (1.7 nm). Yohimbine (76 nm) was injected intrathecally 20 min before the intrathecal administration of ST-91 (2.6 nm), together with ketorolac (3.5 nm). Caffeine (2.0 \( \mu \)m) and norepinephrine (20 nm) were coadministered with the combinations: L-PIA (0.7 nm) with ketorolac (5.2 nm) and U50488H (158 nm) with ketorolac (5.2 nm), respectively.

Statistical Analysis

The time-response data are presented as the number of flinches for the periods of 1–2 min and 5–6 min, and at 5-min intervals thereafter up to 60 min; these data are expressed as mean \( \pm \) SEM flinches per minute. Dose-response curves are presented as the sum of flinches for each observation period. The different treatment groups were examined by one-way ANOVA with a Dunnett's test (\( P < 0.05 \)) for multiple comparisons, provided that the \( F_{\text{ratio}} \) gave \( P < 0.05 \). The dose-response lines were fitted using least-squares linear regression, and the \( ED_{50} \) and 95% confidence intervals were calculated according to Tallarida and Murray.\textsuperscript{37}

Results

Formalin Test and General Behavior

Subcutaneous formalin injection resulted in a highly reliable flinching response with two distinct phases, as indicated in the typical time-versus-effect curve shown in figure 1. The magnitude or time course of behavioral activity did not differ in the control group over the time study, or between the different treatments employed as control vehicles for the respective drug groups (intrathecal saline or 5% cyclodextrin; one-way ANOVA, \( P > 0.05 \)). The control experiments were, therefore, pooled and employed as a common control group.

The intrathecal administration of ketorolac (2.7–80 nm), morphine (0.9–30 nm), ST-91 (1.2–12 nm), U50488H (22–320 nm), and L-PIA (0.3–1.0 nm) did not produce any detectable effect on motor function or general behavior during the observation period (60 min). However, at higher doses, L-PIA (3.0 nm) produced a transient motor dysfunction in all animals, which limited the study of the maximal achievable effect of this compound. The reductions in the incidence of flinching behavior produced by the doses of agonists

Anesthesiology, V 79, No 2, Aug 1993

![Graph](http://anesthesiology.pubs.asahq.org/pdfaccess.ashx?url=/data/journals/jasa/931314/ on 12/22/2018)
employed in the dose-response studies are, therefore, not attributable to motor impairment. None of the antagonists, naloxone (2.8 μg/kg, IP), yohimbine (76 nm, intrathecally), norBNI (20 nm, intrathecally), nor caffeine (2 μg, intrathecally), produced any effect on general behavior or motor function.

Dose-Response Studies

Intrathecal morphine and ST-91, but not U50488H and L-PIA, resulted in a complete dose-dependent suppression of the first phase evoked by formalin (fig. 1, table 1). Ketorolac produced a limited (to approximately 50%), but dose-dependent, inhibition of the first phase of the formalin response (table 1). The second phase of the formalin test was suppressed in a dose-dependent fashion by all of the agents (fig. 2), but with different degrees of maximal effect. As with the first phase, morphine and ST-91 completely blocked the flinch behavior in the second phase formalin test. In contrast, U50488H, L-PIA, and ketorolac each resulted in a dose-dependent inhibition. For ketorolac and U50488H, a plateau effect was observed. The plateau effect is defined when two doses separated by a half log unit produce effects that differ by less than 10%. In this case, the lower of the two doses is defined as the plateau dose. For L-PIA, a submaximal effect is reported, and this reflects the fact that higher doses could not be employed because of motor dysfunction (table 1). The rank order of potency (according to the ED50 values [nmol; table 1]) in the study were as follows: 1) for phase 1 (0–9 min): morphine (3.7) > ketorolac (24) > U50488H = L-PIA = 0; and 2) for phase 2A (10–39 min): L-PIA (0.77) > morphine (3.7) ≥ ST-91 (4.0) ≥ ketorolac (5.2) > U50488H (158).

Isobolographic Analysis of Intrathecal Agents with Ketorolac

Isobolographic analysis, using dose ratios based on the ED50 values of phase 2A of the formalin response, revealed a significantly multiplicative (greater than additive) interaction between ketorolac and morphine, as well as ketorolac and ST-91, in both the first and second phase of the formalin response (fig. 3). The experimental doses are significantly less (Student’s t test, P < 0.05) than the calculated additive doses (fig. 3), as indicated by total dose fractions of less than 1 (table 2). In contrast, ketorolac, in combination with U50488H or L-PIA, resulted in an experimental ED50 value that was not different (P > 0.05) from the theoretical additive point (fig. 4), with a total dose fraction close to 1 (table 2), indicating an additive interaction.

Antagonist Studies

The intrathecal injection of naloxone (2.8 μg/kg, intraperitoneally); yohimbine (76 nm, intrathecally); norBNI (20 nm) or caffeine (2 μg, intrathecally), had no effect on the baseline phase 2 response, and did not affect the inhibition of the formalin response observed with the intrathecal injection of ketorolac (1.7 nm) alone (figs. 5 and 6). In contrast, each antagonist produced significant reversal of the effect produced by the respective agonist: morphine (1.2 nm, intrathecally); ST-91 (2.6 nm, intrathecally); U50488H (215 nm); or L-PIA (2.6 nm). Significant antagonism was also observed when the antagonists were given in the presence of the respective agonist and ketorolac (1.7 nm, intrathecally). The single exception was observed on coadministration of caffeine (2 μg), L-PIA (0.7 nm), and ketorolac (5.2 nm) (fig. 6B).

Table 1. ED50 Values with 95% Confidence Intervals (CI) and % Maximal Inhibition of Intrathecal Nonstimulatory AntiInflammatory Drugs and Antinociceptive Agonists on the Flinching Response Evoked by Formalin Injection

<table>
<thead>
<tr>
<th>Agent</th>
<th>ED50 Values* (nmol) and 95% CI %</th>
<th>Maximal Inhibition ± SEM†</th>
<th>Dose (nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phase 1</td>
<td>Phase 2A</td>
<td>Phase 1</td>
</tr>
<tr>
<td>Ketorolac</td>
<td>24 (13–45)</td>
<td>5.2 (3.2–8.3)</td>
<td>52 ± 8</td>
</tr>
<tr>
<td>Morphine</td>
<td>3.7 (1.6–8.1)</td>
<td>3.8 (2.6–5.4)</td>
<td>85 ± 12</td>
</tr>
<tr>
<td>ST-91</td>
<td>4.1 (2.4–6.9)</td>
<td>4.0 (3.0–5.3)</td>
<td>75 ± 9</td>
</tr>
<tr>
<td>U50488H</td>
<td>—</td>
<td>158 (90–275)</td>
<td>36 ± 12</td>
</tr>
<tr>
<td>L-PIA</td>
<td>0.77 (0.41–1.5)</td>
<td>4 ± 16 (0.81–6.5)</td>
<td>2.8†</td>
</tr>
</tbody>
</table>

* Effective dose producing 50% reduction of the control response in the formalin test.
† ‰ Percentage maximal inhibition (max + 100%) presented as mean ± SEM for 4–6 rats, of the highest dose that resulted in total suppression of the formalin response or did not differ more in effect than 10% from the immediate previous dose.
‡ A slight motor dysfunction was observed in 100% of the animals at this dose.

Anesthesiology, V 79, No 2, Aug 1993
SPINAL NSAID INTERACTIONS

![Graph showing Phase 1 and Phase 2A graphs with different NSAIDs compared to control.](image)

Fig. 2. Dose-response curves for intrathecal morphine, L-PIA, ST-91, ketorolac, and US0488H, presented as the cumulative number of formalin evoked flinches during phase 1 (0-9 min; top) and phase 2A (10-39 min; bottom) on the formalin test. Each dose point on the graph represents the mean ± SEM from 4-6 rats, except for the control group, which includes 20 animals.

Discussion

The formalin test reflects, as does any acute injury state, a surprisingly complex series of events. The initial afferent barrage generates an augmented state of spinal processing, as well as a subsequent phase in which the ongoing afferent input evokes an exaggerated response. In the current study, both the phase 1 and 2 responses were attenuated in a dose-dependent fashion by morphine (μ opioid agonist) and ST-91 (α2 adrenergic agonist), although US0488H (κ opioid agonist), L-PIA (adenosine A1 agonist), and ketorolac (cyclooxygenase inhibitor) had limited effect on phase 1, but resulted in a powerful, though incomplete, suppression of the second phase formalin response. The effects were antagonized by the respective antagonists: naloxone (morphine), yohimbine (ST-91), norBNI (US0488H), and caffeine (L-PIA). The differences in action are further reflected by the different characteristics of the interaction with ketorolac, in which the μ and α2 agents displayed a significant synergy, in contrast to the κ and A1 agonists, with which a simple additivity was noted.

Mechanisms of Antinociceptive Action of Spinal Agonists

Evaluation of the mechanisms of action for the four classes of agents reveals several points of comparison. First, while all agents have higher levels of their respective binding in the dorsal than in the ventral horn, μ and α2 binding sites are significantly reduced by dorsal rhizotomies or capsaicin, although κ and A1 binding are not. Second, consistent with the presynaptic location of binding, μ and α2 agonists produce a dose-dependent, pharmacologically specific reduction in the depolarization-evoked release of C-fiber peptides, although neither adenosine nor κ agonists alter the evoked release of spinal substance P. Third, consistent with the presence of nonafferent binding sites, all agents appear able to diminish the excitation of dorsal horn neurons by a direct postsynaptic action. The concurrent effect on small afferent excitatory input and the postsynaptic projection neurons indicates that a potent and selective reduction in the excitation evoked by C-fiber input can be achieved at drug concentrations that have a minimal effect on other spinal sensory-motor systems. Moreover, to the extent that a pain state possesses a facilitatory component, the ability to block C-fiber transmitter release should prevent the development of the augmentation in spinal processing that occurs with repetitive C-fiber input. On the other hand, the ability of A1 and κ agonists to primarily alter the second phase raises the possibility that the mechanisms whereby these agents work do not involve a blunting of the C-fiber drive, but reflect a moderation of the excitability of the second-order neuron, perhaps by a direct hyperpolarization. These differences appear likely to account for the high therapeutic ratio of spinal μ and α2 agonists, as compared with κ and adenosine agonists in animal models, and may account for the modest effect of the latter agents on the acute (phase 1) formalin pain response.

Mechanism of Action of Spinal NSAID

We have previously shown that spinal administration of several NSAIDs results in a dose-dependent inhibition of the second phase of the formalin test, at doses that are 100-800 times lower than those required after sys-
temic administration, and in a manner that correlates with their capacity to inhibit the synthesis of prostaglandins.\textsuperscript{13} It has also been shown that high-threshold afferent input can evoke release of spinal prostaglandins,\textsuperscript{14–16} and increases in spinal prostaglandin levels can augment Ca\textsuperscript{2+} conductance, enhance C-fiber transmitter release,\textsuperscript{47} and evoke a behavioral hyperalgesia.\textsuperscript{46–51} These joint observations offer convergent support for the hypothesis that cyclooxygenase products play an important role in the regulation of spinal nociceptive processing, leading to a centrally mediated state of hyperalgesia, and that cyclooxygenase inhibitors may play a significant role in altering hyperalgesia by a spinal site of action. Failure of spinal NSAIDs to completely block the phase 2 response reflects the facilitatory role played by cyclooxygenase products in processing the comparatively low level of C-fiber input generated in the formalin-injected paw during this second phase.\textsuperscript{5}

**Interaction Between Spinal Ketorolac and Receptor Selective Agonists**

In the current study, intrathecal ketorolac, combined with either morphine or ST-91, resulted in a significant synergistic effect on both the first and the second phase of the formalin test. The combinations were able to totally block the phase 1 and 2 behavior response at doses that, for the NSAID alone, had no effect on either phase and, for the agonists alone, only resulted in a small reduction (<20%) of the response to the formalin injection. The antagonist studies emphasize that the interaction required the concurrent activation of the respective spinal receptor and the presence of NSAID. Failure of the several antagonists to alter the actions of

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**Table 2. Effect of Intrathecal Ketorolac With Each of the Antinociceptive Agonists, Morphine, ST-91, US0488H, and LPIA, Coadministered in a Fixed-dose Ratio Based on Their Respective ID_{50} Values on Phase 2A (10–39 min) of the Formalin Response**

<table>
<thead>
<tr>
<th>Drug Combination</th>
<th>Dose Ratio</th>
<th>ED_{50} (nmol) and 95% Confidence Interval (of total combination dose)</th>
<th>Total Fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morphine + ketorolac</td>
<td>1:1.4</td>
<td>0.56 (0.4–1.0)</td>
<td>0.64 (0.28–1.2)</td>
</tr>
<tr>
<td>ST-91 + ketorolac</td>
<td>1:1.3</td>
<td>2.6 (1.7–3.8)</td>
<td>2.5 (2.0–3.1)</td>
</tr>
<tr>
<td>US0488H + ketorolac</td>
<td>30:1</td>
<td>128 (74–219)</td>
<td>—</td>
</tr>
<tr>
<td>LPIA + ketorolac</td>
<td>1:6.7</td>
<td>—</td>
<td>2.7 (2.1–3.3)</td>
</tr>
</tbody>
</table>

Anesthesiology, V 79, No 2, Aug 1993

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Fig. 3. Isobolograms showing the interaction between morphine and ketorolac on the phase 1 (A) and phase 2A (B), and the interaction between ST-91 and ketorolac on the phase 1 (C) and phase 2A (D), on the formalin test. In all isobolograms, the experimental dose combination is found significantly (P < 0.05) below the additive point, indicating a synergistic interaction. Each point on the graph represent ED_{50} values ± SEM (shown by heavy lines for the individual agents) from dose-response curves including 16–20 rats.
Fig. 4. Isobolograms for the interaction between US0488H and ketorolac (A) and L-PIA and ketorolac (B) on the phase 2A (10–39 min) of the formalin behavior. The experimental points were not significantly different from the calculated additive points, indicating an additive interaction. Each point on the graph represents ED$_{50}$ values ± SEM (indicated by thick lines for the single agents) from dose-response curves including 12–16 rats.

Ketorolac, along with the complete antagonism of the interactive effects of morphine or ST-91, emphasizes the fact that the interaction was a true synergy, with complete loss of activity by antagonism of only one of the elements of the combination.

The magnitude of the interaction between the NSAID and the µ and α$_2$ agonists can be appreciated by examination of the fractional dose scores. Previous studies with other systems known to be synergistic, such as µ and delta receptors, or µ and α$_2$ receptors, have considered dose fractions of around 0.5 to be significant.$^{33,52}$ With morphine, in the current study, the dose fraction was on the order of 0.1. The magnitude of this synergy was unexpectedly high, and emphasizes the powerful interaction that exists between µ opioid agonists and NSAIDs. The relationship observed between the NSAID and the µ and α$_2$ agonists does not appear to be characteristic of all drug combinations. Thus, in contrast to the µ and α$_2$ interactions, ketorolac interacted in, at best, an additive fashion with either US0488H or L-PIA. Further confirmation of the additive characteristics of the interaction is provided by the observation that, unlike the α$_2$ and µ antagonism studies, the antagonism of US0488H resulted in a subtractive effect. Surprisingly, in the adenosine receptor antagonist studies, caffeine, at a dose that antagonized L-PIA alone, failed to affect the combination of L-PIA and ketorolac. Instead, the combination of caffeine, L-PIA, and ketorolac resulted in a suppression of the second phase of the formalin response. Further studies are required to systematically examine a possible spinal interaction between caffeine and NSAIDs. Clinical studies of postpartum pain$^{53,54}$ and oral surgery pain$^{55}$ have demonstrated that caffeine potentiates the effect of NSAIDs.

Mechanisms of Spinal Drug Interactions
It has long been thought that NSAIDs act in the periphery to diminish afferent input generated by an inflammatory stimulus. The studies on the spinal actions of NSAIDs clearly emphasize that this is not the sole mechanism. Moreover, if the effects of spinal NSAIDs were a general effect on sensory processing, then we would predict that the NSAID interaction with a variety of centrally acting analgesics (including µ/α$_2$/κ/adenosine A$_1$) should be similar. The observation that the spinal interaction of an NSAID (ketorolac) with µ and α$_2$ agonists was synergistic, while the interaction observed with US0488H or L-PIA was merely additive, indicates a more complicated action of spinal NSAIDs. Currently, we believe that four alternative hypotheses may be considered.

Spinal Noradrenergic Terminals. Spinal noradrenergic terminals may exert inhibitory influence on spinal nociceptive processing. It has been suggested that spinal prostaglandins may exert a presynaptic inhibition of the release of noradrenaline from these terminals. Thus, NSAIDs may augment the spinal noradrenergic terminal activity by blocking a prostaglandin-mediated inhibition of this spinal noradrenaline release.$^{49}$ The involvement of noradrenergic pathways in NSAID-antinociception is tenable in light of studies showing a synergistic interaction between the spinal α$_2$ and µ agonists.$^{52,55,57}$ However, synergistic interactions have been found between a spinal α$_2$ agonist (clonidine) and the adenosine agonist NECA.$^{58}$ If the mechanism underlying the interaction in the current study was caused by descending pathways, it would be anticipated that a synergistic interaction would be found between the NSAID and L-PIA.

Pharmacokinetic Factors. Although redistribution of the drugs was not studied, it seems unlikely that the synergistic interaction between ketorolac and morphine or ST-91 depends on altered clearance of either drug. Thus, 10 min after intrathecal administration of the combinations, during the first phase of the formalin test, the behavior evoked by the formalin injection was totally abolished by the combinations at doses of the agents that,
stimulatory G-protein, resulting in increased intracellular cyclic AMP.59 Also, PGI2 and PGD2 are linked to activation of adenylate cyclase.59 Mu opioid and α2 adrenergic receptors are linked to inhibitory G-proteins, which couple the opioid receptor to the K+ channel, resulting in increased K+ conductance and hyperpolarization. This hyperpolarization leads, indirectly, to an inhibition of Ca2+ entry during the action potential.18 Kappa receptors, in contrast to μ and α2 receptors, can directly inhibit Ca2+ entry by a second messenger-linked mechanism.60 A1 adenosine receptors act through inhibitory G-proteins and decrease adenylate cyclase activity. However, the final result seems to be

alone, had no effect at all. Additionally, the synergistic effects were readily reversed with the antagonists.

Second Messengers and Membrane Effects. The action of two agents on different second messenger systems within the same cell may mediate a synergistic interaction. Based on an effect of NSAIDs on prostanoid biosynthesis, and NSAIDs' role as cyclooxygenase inhibitors after spinal administration,45 decrease of spinal prostanoid synthesis is probably the major mechanism for the action of NSAIDs in the spinal cord. Prostaglandin E2 receptors activate adenylate cyclase through a

Anesthesiology, V 79, No 2, Aug 1993

Fig. 5. Effect of the antagonists naloxone (A) and yohimbine (B) on phase 2A (10–39 min) of the formalin test. The bars presents the mean ± SEM (4–6 rats in each group) of the cumulative number of formalin evoked flinches during phase 2A. (A) The darker bars represent (from the left): intrathecal saline (10 μl), morphine (50 nm), ketorolac (37 nm), and the combination morphine (1.2 nm) and ketorolac (1.7 nm). The lighter bars demonstrate the effect of naloxone (2.8 μg/ml) on the agents listed above. Naloxone was injected intraperitoneally 5 min before the formalin injection; morphine, ketorolac, and the combination were injected 10 min before formalin injection. (B) From the left, shown as dark bars, are the following: intrathecal saline (10 μl), ST-91 (4.0 nm), ketorolac (27 nm), and the combination ST-91 (2.6 nm) and ketorolac (3.5 nm), all injected 10 min before formalin. Yohimbine (76 nm) was injected intrathecally 30 min before formalin injection. *Statistical significant reversal of the agonists effect by the antagonist (P < 0.05, one-way ANOVA).

Fig. 6. Antagonist effect of norBNI (A) and caffeine (B) on phase 2A (10–39 min) of the formalin test. (A) Dark bars, from the left, demonstrate the effects of intrathecal saline (10 μl), U50488H (215 nm), ketorolac (27 nm), and the combination of U50488H (156 nm) and ketorolac (5.2 nm). White bars represent the agents listed above coadministered with norBNI (20 nm) 10 min before formalin. (B) From the left, shown as dark bars, are: intrathecal saline (10 μl), LP1A (2.6 nm), ketorolac (27 nm), and the combination LP1A (0.7 nm) and ketorolac (5.2 nm). The lighter bars next to each drug represent the drugs coadministered with caffeine (2 μl) 10 min before formalin injection. The bars represent the cumulative number of formalin-evoked flinches during phase 2A (10–39 min) presented as the mean ± SEM of 4–6 rats in each group. *Statistical significant antagonism of the agonist/NSAID effect using one-way ANOVA (P < 0.05).
similar to the other receptors, with an increase in K⁺ conductance followed by hyperpolarization. Based on the above similarities, we cannot find any ready explanation for the observed synergism based on a postsynaptic second messenger system activated by the different receptors.

**Pre- and Postsynaptic Spinal Actions.** A defining variable that appears to stand out from these studies is that agents possessing a strong presynaptic action on C fibers (morphine/ST-91) displayed synergy with ketorolac, but those without that interaction (U50488H/L-PIA) did not. Under normal conditions, the analgesic effects of μ and α₂ agonists may largely be mediated by the presynaptic action on afferent transmitter release. Prostanoids appear to augment afferent calcium currents and enhance the release of C-fiber primary afferent peptides. We offer the straightforward speculation that the nonlinear augmentation of the effects of μ and α₂ agonist activity by spinal NSAIDs reflects on the augmented terminal excitability otherwise induced by the locally released prostaglandins. The augmentation of this release would counter the known suppressive effect on terminal release. Blockade of that augmentation would substantially enhance the apparent potency of these classes of agents.

**Clinical Significance**

These studies make two points. First, a significant component of human postinjury pain may reflect not only the acute afferent drive evoked by the injury, but also the augmented processing generated by the previous activity in C-fibers. Well defined human psychophysical studies have unequivocally shown that large areas of surprisingly prolonged (3 h), secondary hyperalgesia are evoked by short-term (20 min) activation of even discrete populations of cutaneous C-fibers. Animal studies have shown that these components of pain processing have a unique pharmacology. To that degree, it becomes possible that agents not considered to be strong “analgesics” may effectively diminish the hyperalgesic component of the postinjury pain state. Examples include the NSAIDs and, possibly, other agents, such as nitric oxide, acting in the cascade known to be evoked by repetitive afferent input.

Second, the current work emphasizes a powerful interaction between NSAIDs and certain receptor-selective analgesic agents. The potent synergistic interaction of NSAIDs with certain opioid and nonopioid classes of analgesics supports the use of this class of agents in managing a variety of pain states generated secondary to afferent fiber input. The observation that NSAIDs are only additive with U50488H brings up the interesting consideration that all analgesics, and most notably those that are proposed to act at the k opioid receptor (e.g., butorphanol), may not display a synergistic interaction. The rationale for administration of an opioid with a NSAID is to diminish the dose that is necessary to produce a given therapeutic endpoint and accordingly reduce the respective side effects associated with the use of either class of agents (e.g., respiratory depression and sedation versus gastrointestinal irritation and impaired hemostasis). Clinical demonstration of the magnitude of the efficacy of NSAIDs in reducing μ and α₂ analgesic doses requires systematic human studies employing dose-ratio paradigms. As a final comment, toxicology for spinal NSAIDs in general, and ketorolac in particular, have not been considered. Consequently, spinal delivery of these agents in humans must be considered to be inadvisable at this time.

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Anesthesiology, V 79, No 2, Aug 1993


