Effect of continuous spinal remifentanil infusion on behaviour and spinal glutamate release evoked by subcutaneous formalin in the rat

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Summary
Injection of formalin into the hind paw of the rat evokes a biphasic nociceptive behavioural response, which is considered to be an animal model of postoperative pain in humans. The initial response (phase 1) is caused by activation of peripheral nociceptors and is followed by a second phase attributed to ongoing activity in primary afferents and increased sensitivity of dorsal horn neurones. The latter effect is thought to result from glutamate-mediated N-methyl-D-aspartate receptor activation. In studies to date it has been difficult to discriminate mechanisms underlying phase 1 and phase 2 events because of the long-lasting half-times of intrathecally administered opioids. To further understanding of the opioid pharmacology of the two different phases of the formalin test, we have studied behavioural activity and spinal glutamate release after intrathecal administration of remifentanil, a new short-lasting μ opioid. Intrathecal remifentanil 3 μg μl\(^{-1}\) min\(^{-1}\) delivered during phase 1 inhibited behavioural response during phase 1 (100%), but did not abolish subsequent phase 2 behavioural activity completely (67 (12) %). Intrathecal remifentanil administered separately in phase 1 and phase 2 revealed a similar ED\(_{50}\) (0.2 μg μl\(^{-1}\) min\(^{-1}\)) for inhibition of the behavioural responses. In vivo, spinal microdialysis showed incomplete reduction in glutamate concentrations in response to intrathecal remifentanil administration; this in turn inhibited phase 1 behavioural responses. Therefore we contend that supramaximal doses of intrathecal remifentanil sufficient to inhibit phase 1 activity still permitted sufficient glutamate release to allow spinal facilitation. Incomplete suppression of spinal excitatory neurotransmitter release by intrathecal opioids is consistent with spinal wind-up that is triggered during phase 1 and results in phase 2 afferent drive. This might reflect one of the mechanisms underlying postoperative pain. (Br. J. Anaesth. 1998; 80: 348–353)

Keywords: pain, experimental; analgesics opioid, remifentanil; rat

The formalin test as a preclinical model of tonic persistent inflammatory pain is a well established model of postoperative inflammatory pain licking.\(^1\)\(^2\) Subcutaneous injection of formalin into the paw evokes a biphasic behavioural response: phase 1 starts immediately after formalin injection and is characterized by paw flinching and licking. After a short period of quiescence this is followed by a second phase of paw flinching and licking.\(^1\)\(^2\)\(^4\) Intrathecal delivery of N-methyl-D-aspartate (NMDA) receptor antagonists before injection of formalin, but not between phases 1 and 2, diminished markedly phase 2. This observation has led to the suggestion that phase 2 depends on the acute release of the excitatory neurotransmitter glutamate from neurones during the initial afferent barrage.\(^4\)\(^5\) Indeed, in previous work by Malmberg and Yaksh in conscious rats using an intrathecal loop dialysis system, it was demonstrated that glutamate concentrations increased acutely during phase 1, but not during phase 2.\(^5\) In this study intrathecal morphine delivered before phase 1 reduced behavioural response during phase 1 and continued to suppress phase 2 behaviour even though opioid receptor antagonism was accomplished between phase 1 and phase 2.\(^6\) A major limitation of this study was the use of naloxone to confine opioid activity to the time period of phase 1. Thus, interfering interactions with other endogenous transmitter systems in inflammatory pain mechanisms would also be lost. In a previous study we were able to demonstrate rapid onset of analgesia by intrathecal administration of the new short-lasting μ opioid, remifentanil, within 12 min after the start of a continuous infusion followed by a concomitant fast rate of recovery within 7 min after termination of the infusion.\(^13\) Therefore, a more precise test to examine transient activation of the spinal opioid receptor can be achieved using this new μ opioid which, because of its rapid metabolism, allows opioid activity to be confined to phase 1 of the formalin test.\(^14\)\(^15\) Moreover, we speculate that doses of intrathecal remifentanil that suppress behavioural responses in phase 1 should result in concurrent inhibition of glutamate release.

Materials and methods
The study was approved by the Institutional Animal Care Committee of the University of California, San Diego. Rats (male Sprague–Dawley, Harlan Indus-
trials, Indianapolis, IN, USA; 300–350 g) were implanted with intrathecal catheters and thereafter kept in separate cages on a 12-h light–dark cycle and given water and food ad libitum. Each rat was implanted as described below with an intrathecal catheter or microdialysis system, or both. Animals were allocated randomly to treatment groups and were used only once. The animals were killed after the experiment was completed.

ANIMAL PREPARATION

Before surgery animals were anaesthetized with 2–3% halothane and 50% oxygen in air, breathing spontaneously via a mask. Surgical insertion of the intrathecal catheters was performed using a modified version of techniques described previously. Intrathecal catheters were made of polyethylene tubing (PE-10, id 0.28 mm, od 0.61 mm) tied at one end into a small knot for fixation under the skin. After shaving and preliminary cleaning of the surgical area with alcohol and betadine, the rat was placed in a stereotaxic apparatus. After incision of the atlanto-occipital membrane, the intrathecal catheter was inserted into the intrathecal space and passed to the rostral edge of the lumbar enlargement, 8.5 cm posterior to the cisterna magna. The external arm of the catheter was then tunnelled subcutaneously and fixed under a skin suture. Only animals with normal motor functions were used in the experiments.

BEHAVIOURAL TESTING: FORMALIN TEST

For behavioural testing conscious rats were connected to different intrathecal catheters (see below) and placed in round Plexiglass cages (9 × 35 cm) on top of a temperature-maintained glass surface (30 °C). A mirror was set up to improve visibility of the animal’s behavioural responses. An adaptation period of 15 min was allowed, and then each animal was picked up gently by another investigator and 50 μl of 5% formalin were injected s.c. into the right hind paw. The animal was replaced in its cage. Flinches of the right hind paw without concomitant movement of the left hind paw or stepping motion were counted as behavioural responses. An adaptation period of 15 min was allowed, and then each animal was picked up gently by another investigator and 50 μl of 5% formalin were injected s.c. into the right hind paw. The animal was replaced in its cage. Flinches of the right hind paw without concomitant movement of the left hind paw or stepping motion were counted as behavioural response and were evaluated at 1–2, 5–6, 9–10 and 14–15 min, and continued at 5-min intervals up to 60 min after injection of formalin. The interval from 0–10 min was defined as phase 1 of the formalin test (acute C-fibre stimulation) and the interval from 10–60 min was defined as phase 2.

CONSTRUCTION OF MICRODIALYSIS CATHETER

Intrathecal loop dialysis catheters were constructed from hollow fibres (200 μm id, 300 μm od, 11 kDa molecular weight cut-off; Filtran, AN 69-HF). An 18-cm length of fibre was coated with a thin layer of epoxy (Devcon Corp., Danvers, MA, USA) except for a 4-cm portion in the middle. Nichrome-Formvar wire (0.0026 mm; A-M systems, INC., Everett, WA, USA) was passed through the fibre and both ends of the fibre were attached to pieces of silicon PE-10 (3.5 cm length) with cyanoacrylate (Borden, Inc., Columbus, OH, USA). The fibre was then bent such that the uncoated portion formed a “U”-shaped loop. Finally, both pieces of silicon PE-10 were bound together with silicon rubber at the level of their junction with the dialysis fibre.17,18

IMPLANTATION OF THE CATHETER

To implant the microdialysis catheter, a technique similar to that described for placement of intrathecal catheters was used. After the surgical procedures, a recovery period of 5 days was allowed before initiating sampling and testing of dialysate. The neurological status of the animal was assessed after surgery and before dialysate testing; neurological status was evaluated after flushing the intrathecal catheter with 10 μl of normal saline. Animals with impaired motor function were killed.

PERFUSION OF CATHETERS

To initiate dialysis, one of the externalized silicon PE-10 connections was attached to a 50-cm length of PE-10 tubing (inflow) and the other arm to a 50-cm length of PE-10 (outflow). A syringe pump (Harvard Compact Infusion Pump, Model 975, MA, USA) fitted with a 5-cc plastic syringe was used to perfuse the dialysis tubing with artificial cerebrospinal fluid (ACSF) at a rate of 10 μl min⁻¹. ACSF contained (nmol litre⁻¹): Na⁺ 151.1, K⁺ 2.6, Mg²⁺ 0.9, Ca²⁺ 1.3, Cl⁻ 122.7, HCO₃⁻ 21.0, HPO₄²⁻ 2.5 and glucose 3.5. ACSF was bubbled with 95% oxygen–5% carbon dioxide before each experiment to adjust the final pH to 7.2. All experimental manipulations were preceded by a 30-min washout period and collection of two control samples (10 min each).17

SAMPLE COLLECTION AND ANALYSIS

Dialysate samples were collected on ice and kept frozen at −20 °C until analysed for amino acids. All dialysis samples were analysed routinely for glutamate content using the PITC (phenyl isothiocyanate) derivatization procedure and a Waters HPLC with a reverse phase C18 column (3.9 × 300 mm, 4-μm particle) and UV detector. Amino acid content was measured in single 25-μl aliquots of dialysate. Methionine sulphone was added to each dialysate sample as an internal standard. Sensitivity was 5–10 pmol/ injection. Amino acid peak heights were normalized initially to the methionine sulphone peak and then quantified based on a linear relationship between peak height and amounts of corresponding standards. External standards were run daily.

FORMALIN TEST AND INTRATHECAL PERFUSION IN AWAKE ANIMALS

Formalin was injected s.c. into the hind paw after the microdialysis system was perfused for 30 min (washout period) and two baseline samples (10-min duration each) were collected. After injection, samples were collected at 10-min intervals until 20 min after injection and then at 20-min intervals after injection (samples until 10 min = phase 1, samples from 10 to 60 min = phase 2).

DRUGS

Drugs given intrathecally in this study were remifentanil HCl (molecular weight = 369) (Glaxo, Research
Triangle Park, NC, USA) and normal saline as control. Intrathecal remifentanil infusion rates are expressed in μg μl⁻¹ min⁻¹. Intrathecal injection was achieved by connecting the animal to a microinjection syringe pump (Harvard Instruments) before testing via a length of calibrated PE-50 tubing. Intrathecal drug was injected at a flow rate of 1.0 μl min⁻¹. Thus the lowest total amount of intrathecal remifentanil was 0.4 μg for the phase 1 infusion of 0.02 μg μl⁻¹ min⁻¹; the highest amount for phase 1 (3 μg μl⁻¹ min⁻¹) was remifentanil 60 μg.

In a separate experiment, animals receiving a supramaximal dose of intrathecal remifentanil for phase 1 suppression were given naloxxone (Dupont Pharmaceutical, Wilembur, USA) 0.5 mg/kg body weight i.p., 10 min after injection of formalin. This experiment served as a control for residual intrathecal remifentanil activity affecting phase 2. All drugs were dissolved in sterile normal saline.

STATISTICAL ANALYSIS

Behavioural data are expressed as mean (SEM) number of flinches per minute for 1, 5, 10, 15 min, and at 5-min intervals up to 60 min. Statistical analysis of the behavioural data was performed using the one-way ANOVA test followed by the Student’s Newman–Keuls multiple comparisons test. For illustration, the dialysate group was calculated as percentage change in baseline values (mean (SEM)). Statistical analysis of glutamate release was performed by the Kruskal–Wallace test. For all statistics, \( P<0.05 \) was considered significant. Dose–response curves are presented as percentage of the maximal possible effect (%MPE). For all drugs, the dose–response analysis, as described by Tallarida and Murray, was performed. \( ED_{50} \) and 95% confidence intervals were calculated using the least squares linear regression model, where the log dose values were used.

Results

INTRATHECAL INFUSION OF REMIFENTANIL BEFORE AND DURING PHASE 1

Injection of formalin resulted in a biphasic incidence of flinching of the formalin injected paw, as demonstrated in the animals receiving intrathecal saline infusion during phases 1 and 2 (fig. 1). Intrathecal infusion of remifentanil during phase 1 and intrathecal saline during phase 2 resulted in complete dose-dependent suppression of phase 1 behaviour (\( P<0.05 \) compared with saline controls).

Intrathecal remifentanil at the highest dose (3 μg μl⁻¹ min⁻¹) during phase 1 resulted in an approximate 40% reduction in the magnitude of phase 2 behaviour compared with the control groups (fig. 2). To emphasize that depression of phase 2 did not result from residual action of remifentanil during phase 2, studies with phase 1 intrathecal remifentanil infusions were repeated with administration of naloxxone 0.5 mg kg⁻¹ i.p., 10 min after injection of formalin. Phase 2 suppression was unaltered, emphasizing that the persistent effects were not caused by residual opioid agonist action during the second phase (see fig. 2).

INTRATHECAL INFUSION OF REMIFENTANIL DURING PHASE 2

Intrathecal infusion of saline in phase 1 and intrathecal infusion of remifentanil during phase 2 revealed no difference in phase 1 behaviour compared with control animals (\( P>0.05 \)), whereas remifentanil blocked phase 2 of the formalin test in a dose-dependent manner (intrathecal remifentanil 0.2, 2.0 and 3 μg μl⁻¹ min⁻¹; \( P<0.05 \) (see fig. 1). The \( ED_{50} \) for remifentanil was similar for suppression of both phases (phases 1 and 2: 0.2 μg μl⁻¹ min⁻¹ (95% CI 0.1–0.4 μg μl⁻¹ min⁻¹)) (fig. 3).
**Remifentanil effects on behaviour and spinal glutamate release in the rat**

Before treatment, measurable glutamate concentrations were present in the spinal dialysate of the unanaesthetized rat with stable baselines for saline control and intrathecal remifentanil groups. Formalin injection resulted in an increase in glutamate concentrations, which was consistently evident during the first 10 min after formalin injection in the intrathecal saline compared with the intrathecal remifentanil group (71 (34) % vs 21 (36) %; \( P < 0.05 \)). This release corresponded to the appearance of phase 1 behaviour. After phase 1, glutamate concentrations decreased and reached baseline values during phase 2 in both groups (fig. 4).

**Discussion**

The formalin test has been used widely as a model of inflammatory pain\(^1,^2\) to investigate the mechanisms and pharmacology of spinal facilitation. Assessment of peripheral afferent activity has emphasized that injection of the irritant formalin leads to an acute barrage of small primary afferents followed by low level, but persistent, afferent activity. The corresponding activity in dorsal horn neurones\(^2^1\) and behaviour sequelae suggests a second phase of behaviour that is unexpectedly prominent given the magnitude of the afferent input during this second phase. The ability to reduce (but not completely block) this second phase by spinal delivery of NMDA receptor antagonists given before formalin injection is believed to reflect the role of glutamate release during this acute interval in triggering the second phase facilitation.\(^2^2\) It was suggested that block of afferent activity by opioids during the first phase should diminish the second phase behavioural response.\(^2^3\) In previous work, we used intrathecal morphine and blocked the opioid receptor during the second phase by delivery of naloxone between phases 1 and 2.\(^2^4\) However, interpretation of these results was hindered by rebound hyperalgesia that can occur after using the antagonist naloxone.\(^2^5\)

Therefore, in our study we were able to address this hypothesis more accurately by the use of the short-lasting opioid agonist remifentanil. This lipid soluble agent is cleared rapidly from the intrathecal space and metabolized in plasma by plasma esterases.\(^1^5,^2^6\) As we have shown previously in the rat model, continuous infusion of remifentanil rapidly
remifentanil, we suggest that spinal μ opioid receptors modulate but do not abolish the mechanisms underlying spinal sensitization in this model of inflammatory pain.

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References


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