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Standardization of the rat paw formalin test for the evaluation of analgesics

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Abstract. Administration of 5% formalin into the rat or guinea pig hind paw evokes two spontaneous responses: flinching/shaking and licking/biting of the injected paw. The temporal and behavioral characteristics of these objective endpoints are described. Additionally, several practical suggestions aimed at standardizing this test for the evaluation of analgesics are presented. The early/*acute* and late/*tonic* (0–10 and 20–35 min post-formalin, respectively) phases of flinching were used to quantitate antinociception in the rat. PD 117302, the kappa selective agonist, was three times more potent than morphine against tonic flinching after SC administration. Formalin may therefore be a noxious stimulus of choice in the evaluation of kappa agonists. Morphine was only twice as potent against tonic flinching as against acute flinching or the tail-dip reflex to water (50° C). In contrast, PD 117302 was 27 times less potent on early phase and was inactive in the tail-dip test. Thus, while morphine is essentially equipotent across tests, PD 117302 shows a spectrum of activity with impressive potency and efficacy being obtained against tonic pain. Kappa receptors may therefore be prominently involved in tonic pain states. Aspirin given orally was not consistently antinociceptive in either phase of the formalin test. Spinal transection completely abolished late phase responding but only partly attenuated flinching in the early phase. This suggests that the relative involvement of spinal (as opposed to supraspinal) processing of noxious inputs may, at least in part, be a function of stimulus intensity and underlie the differences in antinociceptive potency observed in this work.

Key words: Formalin-induced behaviors – Nociception – Tonic pain – Phasic pain – Morphine – PD 117302 – Aspirin – Spinal transection

Traditional analgesic tests with rodents measure transient rather than continuous pain. However, the relevance of high intensity, phasic endpoints to clinical pain has been increasingly questioned. A greater consideration of the physiological processes involved in nociception (particularly in relation to tonic, as compared to phasic, pain states) is necessary if novel analgesics are to be properly evaluated (Dennis and Melzack 1979; Wall 1984a, b; Millan et al. 1988b; Shaw et al. 1988; Parsons and Headley 1989). Several animal models of persistent nociception have been proposed. The most widely characterized of these is adjuvant-induced polyarthritis in the rat (Millan 1986; Colpaert 1987; Besson and Guilbaud 1988). There is no doubt that this model provides indices for pain and hyperalgesia (Calvino et al. 1987; Colpaert 1987; Millan et al. 1987). Nor is there any question that electrophysiological (Lombard and Besson 1989) and biochemical (Cesselin et al. 1980; Höllt et al. 1987; Millan et al. 1987) changes, indicative of tonic activity in endogenous opioid systems, accompany the behavioral manifestations presumed to stem from prolonged nociception. However, induction of polyarthritis results in a whole animal disease characterized by widespread systemic involvement. These effects are of such severity that specific functional, behavioral and morphological changes in the nervous system cannot confidently be regarded as a consequence of increased nociceptive input alone. It has therefore been recognized, on both scientific and ethical grounds, that alternative models of persistent nociception are highly desirable. Herz and colleagues have developed a related model; namely, unilateral localized inflammation of a hindlimb induced by Freund's adjuvant (Millan et al. 1988a; Stein et al. 1988). Similar work, using a variety of noxious agents, is being pursued by Iadarola et al. (1988). Both groups have demonstrated biochemical changes qualitatively similar to those described following induction of polyarthritis. These include a rapid (within 8 h) elevation in dynorphin mRNA in the spinal cord (Iadarola et al. 1988) followed by a delayed (within 24–48 h) increase in the levels of immunoreactive dynorphin (Iadarola et al. 1988; Millan et al. 1988a). Thus, it seems that changes heretofore associated with prolonged nociceptive states can be elicited by nociceptive stimuli which extend be-

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yond the phasic stage, but do not require a chronic abnormality.

Assessment of behavioral indices in the chronic arthritic rat, and in the other inflammatory models just mentioned, rely on demonstrating hyperalgesia in the inflamed limb in response to noxious heat (Iadarola et al. 1988) or pressure (Millan et al. 1987, 1988a; Stein et al. 1988). We believe, however, that the ideal endpoint should be a simple spontaneous behavior that can be quantified objectively.

Localized inflammation and pain produced by injection of formalin into an animal's paw has been used as a noxious stimulus in several species, for example, rat and cat (Dubuisson and Dennis 1977), mouse (Hunskaar et al. 1985; Murray et al. 1988) and monkey (Alreja et al. 1984). The rat paw formalin test is often associated with cumbersome and subjective rating scales which have probably hindered its widespread use in analgesic testing. We have previously described two objective, spontaneous behaviors of rats induced by formalin which we believe are indicative of pain (Wheeler et al. 1988; see also Ryan et al. 1985). Furthermore, we have recently demonstrated that, when compared to a variety of noxious chemical agents commonly used in the study of inflammation and hyperalgesia, formalin elicits robust, spontaneous behavioral responses (Wheeler-Aceto et al. 1990). We propose that, of the agents tested, formalin causes a behavioral syndrome most widely applicable to the study of acute and tonic pain states and the physiology underlying them. We have assessed the antinociceptive activity of morphine, the prototypical agonist at mu receptors, and PD 117302 in our version of the formalin test. The latter compound has been shown in vitro and in vivo (Leighton et al. 1987) to be a kappa selective opioid agonist which has high affinity at kappa sites (Clark et al. 1988). Aspirin was included as a representative non-steroidal anti-inflammatory agent. The present report formalizes the test for the evaluation of analgesics and presents data which may offer important insights into the differences between acute and tonic pain states.

Materials and methods

Animals

Male Sprague Dawley albino rats (70–90 g, 26–30 days of age; Zivic-Miller Laboratories, Zelienople, PA) and male Dunkin Hartley guinea pigs (200–250 g; Hilltop Lab Animals, Scottdale, PA) were used. One part of the study employed larger rats (200–225 g and 275–325 g, 38–42 and 54–58 days of age, respectively). Animals were housed singly (guinea pigs) or in groups of four to eight (rats) under a standard 12 h light: 12 h dark cycle (light phase 7 a.m. – 7 p.m.) in a room maintained at $22 \pm 1°$ C and approximately 50% relative humidity. Food and water were available ad libitum. Testing was carried out between 11.00 a.m. and 5 p.m. Animals were acclimated to individual Plexiglas observation chambers (L, 21 cm; W, 25 cm; H, 30 cm) for at least 1 h prior to testing. Each chamber contained a thin layer of fresh wood-chip bedding and was visually shielded (neutral red) on all sides except the side facing the observer. Each animal was used only once.

Effect of formalin

After the acclimation period, each animal was injected SC into the dorsal surface of the right hind paw with 50 µl formalin solution (Merck & Co.; 5% in isotonic saline, Murray et al. 1988) or 50 µl saline as previously described (Wheeler-Aceto et al. 1990). This procedure was approved by the IACUC at Temple University.

Behavioral testing. Preliminary experiments with formalin (Wheeler et al. 1988) have revealed two spontaneous behaviors indicative of pain, both of which can be quantitated objectively. The behaviors are i) flinching/shaking of the paw and/or hindquarters, recorded as the number of flinches per observation period; and ii) licking/biting of the injected paw, recorded as total licking time (s) per observation period. Flinching, at its most violent, could equally be described as paw shaking and when less vigorous as paw lifting. It is primarily observed as drawing the paw under the body and rapidly vibrating it; since this causes a shudder or rippling motion across the back it is easy to observe even when the paw itself is not visible. Each episode of shaking or vibrating the paw or each shudder of the back/hindquarters is recorded as one flinch. Licking of the injected paw is self-explanatory. The effect of formalin on overt behavior was assessed by observing animals in pairs (rats) or singly (guinea pigs) and recording spontaneous flinching and licking for 90 min. Results are expressed as mean number of flinches ± standard error of the mean (SE) or mean licking time (s) ± SE occurring in a given time period. Groups of at least six animals were used.

Spinal transection

Surgery. Transections were performed on rats anesthetized with ketamine HCl (75 mg/kg, IP, Ketalar®, Parke-Davis, Morris Plains, NJ). The skin on the back at the level of T6–T9 was shaved and a 1–2 cm incision made. The vertebral arch was exposed by blunt dissection and a laminectomy carried out between T6 and T9. To ensure complete transection, a 1–2 mm section of the exposed spinal cord was removed. The area was then packed with Gelfoam (Upjohn, Kalamazoo, MI) to minimize bleeding. At the end of surgery the incision was closed with 9 mm stainless steel wound clips. Sham-operated animals were anesthetized and received only an incision. Spinally transected rats were closely monitored for 48 h prior to testing. During this time they were bathed in warm water and their urine was expressed manually.

Behavioral testing. The response of sham-operated and transected rats to two forms of noxious stimulus was evaluated:

i) Tail dip test. Animals were held over a water-bath maintained at $48 \pm 0.2°$ C (preliminary experiments revealed this temperature as being most appropriate to distinguish and quantitate decreases in tail-flick latency resulting from spinal transection). The distal 5 cm of the tail was immersed and the latency until the rat curled its tail out of the water was timed. The tail was dried at the end of each trial. A cut-off time of 15 s was chosen to prevent tissue damage. The latency obtained from an initial trial was discarded. The tail-flick time for each rat was calculated as an average of two subsequent trials performed approximately 2 min apart. Latencies were assessed prior to surgery, at 24 h post-surgery, then at 48 h post-surgery immediately before hind paw injection of saline or formalin. In addition, the tail dipping was repeated at approximately 15 min and 55 min after formalin/saline administration.

ii) Nociceptive response to formalin. Flinching and licking behaviors were recorded in both transected and sham-operated rats ($n = 7$ or 8 per group). Observations were made between 0–10 min (early/acute phase) and 20–50 min (late/tonic phase) following injection of 50 µl isotonic saline or 5% formalin into the right hind paw.

Assessment of antinociceptive activity

The potencies of morphine sulfate (Mallinckrodt, St. Louis, MO), PD 117302. HCl ((±)-*trans*-N-methyl-N[2-(1-pyrrolidinyl)-cyclohexyl]benzo[b]-thiophene-4 acetamide, kindly supplied by Parke-Davis, Ann Arbor, MI) and aspirin (Miles, Elkhart, IN) were assessed in both early (0–10 min) and late (20–50 min) phases of the formalin test. Morphine and PD 117302 were dissolved in saline and administered SC; doses are expressed in terms of the free base. A suspension of aspirin was prepared with 0.5% carboxymethylcellulose (CMC) solution (sodium salt, Sigma, St Louis, MO) and given orally to animals which had been fasted overnight. Control animals received either saline (0.2 ml/100 g, SC) or 0.5% CMC (1 ml/100 g, PO). The pretreatment times for all three test agents (Table 3) were chosen so that peak antinociceptive activity coincided with the observation period. Separate groups of rats ($n = 5$–10 per dose) were used to generate dose-response curves in each phase of the response, and for each agent. Data are expressed as mean % antagonism of formalin-induced flinching or licking ± SE, and are calculated for individual drug-treated, formalin-injected rats as follows:

$$\frac{(\text{Mean formalin response} - \text{Mean saline response}) - \text{Individual response}}{(\text{Mean formalin response} - \text{Mean saline response})} \times 100$$

where *mean formalin response* is the mean behavioral score obtained in concurrently run vehicle-treated, formalin-injected (formalin control) rats. *Mean saline response* is the pooled behavioral score from naive rats injected with 50 µl isotonic saline into the hind paw. For example, in the 70–90 g weight range mean scores following injection of saline were 4 ± 1 flinches and 17.3 ± 3.3 s licking between 0 and 10 min ($n = 26$); and 4 ± 1 or 8 ± 1 flinches and 13.7 ± 4.3 or 20.6 ± 5.2 s licking from 20–35 and 20–50 min, respectively ($n = 32$).

Additionally, in order to assess the potencies of morphine and PD 117302 against a phasic end-point, the A_{50} (mg/kg, SC) of each agent was determined in the warm water (50 ± 0.2° C, 15 s cut-off time) tail dip test. Tail withdrawal latencies were recorded as described for the spinal transection study, immediately before dosing and at 20 min after morphine or 30 min after PD 117302. Following SC administration, the latter has been shown to produce rapid (30 min to peak) and long lasting (~ 240 min) antinociception in the rat paw pressure test (Leighton et al. 1987). Results were normalized to % maximum possible effect for each animal where:

$$\%\ \text{MPE} = \frac{(\text{post-drug latency} - \text{pre-drug latency})}{(\text{15-pre-drug latency})} \times 100.$$

To confirm that the antinociception associated with morphine in the formalin test is mediated via opioid receptors, the ability of the enantiomers of naloxone to antagonize the effect of an approximate A_{70} dose of morphine (determined from the flinching dose-response data) was assessed. Both (+)-naloxone HCl (courtesy of Dr. A.E. Jacobson, National Institutes of Health, Bethesda, MD) and (−)-naloxone HCl (Endo Laboratories Inc., Garden City, NY) were dissolved in saline and administered SC at 0.03 and 1 mg base/kg 5 min before (early phase) or 15 min after (late phase) injection of formalin (i.e. in each case, 15 min after morphine).

In a separate series of experiments the effects of repeated dosing with either vehicle or aspirin (125 mg/kg, PO twice daily for 2.5 days, last dose 12 h prior to testing) on formalin-induced behavior and paw edema were determined in the same animals. Paw volume was measured by a standard volumetric technique using a calibrated plethysmometer (Ugo Basile, Varese, Italy). Volumes were taken immediately prior to formalin at $T = 0$ min; between the early and late phase behavioral observations at 18–20 min; at the end of the observation period $T = 60$ min, and then every 30 min up to 3 h post-formalin. For comparison, paw volumes were also measured in separate rats receiving a single dose of aspirin (125 mg/kg, PO) or vehicle 25 min prior to formalin. In addition, the effect of morphine (0.3, 1 and 3 mg/kg, SC at $T = 0$ min) and PD 117302 (0.1, 1 and 10 mg/kg, SC at T = −10 min) on paw volume was assessed at 60 min post-formalin in groups of five rats.

Statistical analyses

Statistical comparisons were made by Student's t test for independent samples (paired or unpaired, where appropriate). Multiple comparisons against control were made by analysis of variance (ANOVA) followed by a Dunnett's t test. Multiple pairwise comparisons were made by ANOVA followed by Scheffé's procedure. Values of t with a probability of less than 0.05 ($P < 0.05$) were considered to indicate a statistically significant difference between means. Antinociceptive potency is expressed as the A_{50} and 95% confidence limits and was determined by linear regression analysis from the percent antagonism of formalin-induced responses and %MPE in the tail-dip test. All statistical analyses were performed with the aid of a statistical package, Statview 512+ (BrainPower Inc., Calabasas, CA), and a Macintosh PC. Relative potencies of morphine and PD 117302 were determined from a parallel line assay (Tallarida and Murray 1987).

Results

Characterization of formalin

Recording of flinching and licking, at 5 min intervals for 90 min post-injection in a large number of animals clearly demonstrated a biphasic response for both behaviors (Fig. 1). An immediate effect (0–7 min) was followed, after a short quiescent period, by a prolonged response which, for flinching, was maximal between 20 and 50 min and persisted for up to 80 min. Tonic licking was of shorter duration (18–38 min), with a peak between 18 and 35 min.

Based on the time course data, 0–10 min post-formalin (early phase) and 20–50 min post-formalin (late phase) were initially chosen as appropriate observation

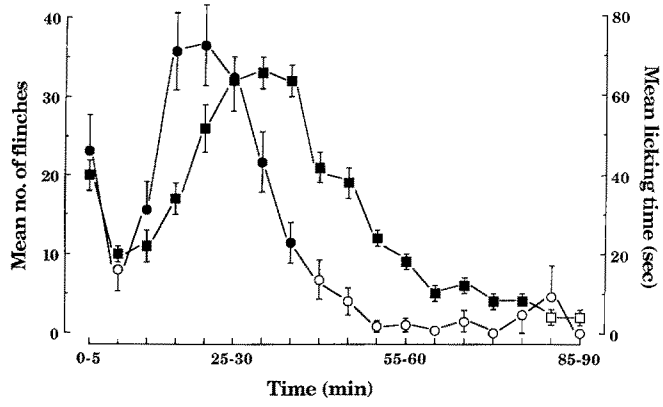

Fig. 1. Time course of flinching and paw licking responses in 70–90 g rats following injection of 5% formalin (50 µl, SC) under the dorsal surface of the right hind paw at T = 0. Each point is the mean behavioral score ± SE ($n = 17$) per 5 min. *Closed symbols* are values which were significantly different from control (isotonic saline, 50 µl SC into the right hind paw, $n = 16$, data not shown), $P < 0.05$, Student's t test for unpaired observations. ■–□ Flinching; ●–○ licking

Table 1. Comparison of early (0–10 min) and late (20–35 min) phase flinching and licking responses in male Sprague Dawley albino rats from three different weight/age groups. Behavior was monitored following injection of 5% formalin (50 µl, SC) into the dorsal surface of the right hind paw

Weight (g)	Age (days)	*n*	Mean no. of flinches ±SE		Mean licking time (s) ±SE	
			0–10 min	20–35 min	0–10 min	20–35 min
70–90	26–30	37	46±3	95±5	63.8±9.2	177.3±11.1
200–225	38–42	8	24±5**	68±16*	30.3±13.3	194.0±29.4
275–325	54–58	8	17±5**	52±6**	41.0±16.3	48.6±12.8**

* $P<0.05$, ** $P<0.01$. Significance was determined with ANOVA followed by Dunnett's *t* test and indicates difference from 70–90 g score

periods to investigate both the phasic and tonic aspects of the formalin response. However, subsequent analysis of the data obtained, across several different time windows, revealed that while a 0–10 min observation period is required to generate a consistent early phase score, a 15 min late phase observation, between 20–35 min post-formalin, is adequate for quantitating antinociceptive activity. During the early phase there are typically 45–50 flinches and 60–65 s licking time. In the 20–35 min observation period there are 90–100 flinches with a licking time of 180–200 s. The remainder of this paper therefore refers to the 0–10 min and 20–35 min intervals, even though all of the rats were actually observed for 20–50 min.

In most of our studies we have used rats weighing 70–90 g, with ease of direct intrathecal administration of analgesics being our primary reason for choosing this size of animal. Few studies of this kind have been performed in rats of this weight and age. We therefore compared the response to formalin in rats from two other commonly used weight ranges, 200–225 g and 275–325 g, with that obtained in 70–90 g animals. The results are summarized in Table 1.

The time course of the response (0–90 min post-injection) was qualitatively similar in all three groups, but some quantitative differences were observed. The flinching response (both early and late phase) gradually diminished with increasing age but, in all cases, remained significantly greater than that caused by injection of isotonic saline in age-matched controls (data not shown). Early phase licking was also reduced, although this effect was not significant. Late phase licking in 200–225 g rats was, however, just as vigorous as that observed in the youngest animals. In contrast, 275–325 g rats exhibited a very much reduced late phase licking response. (In additional studies using rats in the range 370–420 g both phases of licking behavior were much attenuated or absent). Despite generally reduced response levels in older rats, the absolute duration of each phase and behavior was remarkably consistent across all groups.

In guinea pigs, formalin induced the same responses, i.e. flinching and licking, with somewhat different qualities and time course (Fig. 2). After a short delay (15–45 s), guinea pigs responded violently to formalin injection. Indeed, during this stage of the response the high level of behavioral agitation (flinching, licking and flinch jumps) made scoring difficult. The acute phase persisted for 2–3 min before subsiding. In contrast to rats, guinea pigs exhibited no second peak in either behavior. Rather, both flinching (up to 90 min) and licking (up to 50 min) continued at a relatively low level. Despite the low level of activity in this species there was still a striking contrast to animals receiving an injection of saline into the paw, which produced zero behavioral scores.

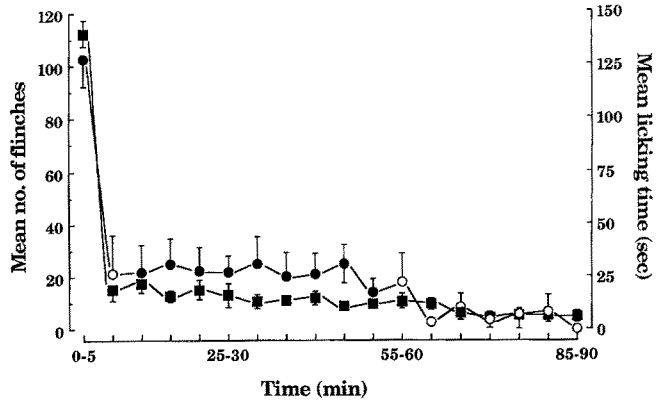

Fig. 2. Time course of flinching and paw licking responses in 200–250 g guinea pigs following injection of 5% formalin (50 µl, SC) under the dorsal surface of the right hind paw at T = 0. Each point is the mean behavioral score ±SE ($n=5$) per 5 min. Animals injected with isotonic saline (50 µl, SC into the right hind paw, $n=5$) had zero behavioral scores in both categories. *Closed symbols* are values which were significantly different from saline control ($P<0.05$, Student's *t* test for unpaired observations). Symbols as in Fig. 1.

Effect of spinal transection

Formalin-induced flinching in the rat tends to be rhythmic in nature. Moreover, unlike licking, it is not a reactive behavior, i.e. no deliberate/directed motor activity is involved. These factors prompted us to establish whether the flinching response is a spinal reflex or if some supraspinal processing is necessary for its generation.

Spinal transection abolished both phases of the licking response (Table 2). However, despite the lack of muscle tone in the hindquarters, a weak foot withdrawal reflex was observed during the first 2 min after formalin injection. While the early-phase flinching response in formalin-injected transected rats was significantly reduced (both qualitatively and quantitatively: 59.1 ± 5.9% fewer flinches) compared to sham-operated animals, it was still significantly greater than that produced in saline injected, transected rats. Late phase flinching was prevented by transection.

Table 2. Comparison of early (0–10 min) and late (20–35 min) phase flinching and licking responses in sham operated or spinally transected (T6–T9) rats. Forty-eight hours after surgery rats received isotonic saline (50 µl, SC) or 5% formalin (50 µl, SC) into the dorsal surface of the right hind paw

Surgery + paw injection	*n*	Mean no. of flinches ± SE		Mean licking time (s) ± SE	
		0–10 min	20–35 min	0–10 min	20–35 min
Sham operated + formalin	8	40 ± 3***	95 ± 7***	71.8 ± 18.3***	172.0 ± 20.4***
Sham operated + saline	8	6 ± 2	3 ± 1	17.6 ± 5.2	4.2 ± 2.7
Spinal transection + formalin	8	16 ± 2**,***	2 ± 1**	3.2 ± 1.1**	0 ± 0**
Spinal transection + saline	7	0 ± 0	0 ± 0	2.0 ± 1.3	0 ± 0

** Significantly different from sham operated score and *** significantly different from saline injected total ($P < 0.01$, ANOVA followed by Dunnett's *t* test)

In the tail dip test, withdrawal times prior to surgery were in the range 3.1–8.8 s (mean 4.8 ± 0.2 s, $n = 31$). At 24 h post-surgery, latencies in sham-operated animals (4.4 ± 0.1 s, $n = 16$) were not changed compared to pre-surgery responses. In transected rats, however, there was a 63 ± 2.6% decrease in the time to tail withdrawal (1.8 ± 0.1 s; $P < 0.001$, Student's paired *t* test, $n = 15$). At 48 h post-surgery, reflex times for sham and transected rats were 4.4 ± 0.2 and 2.1 ± 0.1 s, respectively. Response times recorded at 10 min (4.8 ± 0.2 and 2.6 ± 0.1 s) and 50 min (4.7 ± 0.2 and 2.5 ± 0.1 s) after injection of formalin or saline into the right hind paw were slightly slower than pre-injection reflexes, but the effect was not consistent, nor was it associated with any specific experimental condition, i.e. neither saline nor formalin injection in either transected or sham-operated rats.

Assessment of antinociceptive activity

A_{50} values for morphine and PD 117302 against both early and late phases of formalin-induced flinching are shown in Table 3. These data demonstrate that, in terms of A_{50}, PD 117302 is approximately three times more potent than morphine against late phase flinching (0.2 versus 0.6 mg/kg, SC, or approximately 0.56 and 2.1 µmol/kg, respectively). We also recorded licking behavior in these animals; in our opinion, licking in the rat is a less satisfactory endpoint by which to measure antinociception (see Discussion). The licking data obtained are therefore not included here.

Table 3. A_{50} (mg/kg) and 95% confidence limits for morphine, PD 117302 and aspirin against early and late phase formalin-induced flinching in 70–90 g rats

Compound and route	Pretreatment time (min before formalin)	Phase	Observation period (min)	A_{50} and 95% CL Flinching
Morphine SC	−20	Early	0–10	1.1 (0.9–1.4)
PD 117302 SC	−30	Early	0–10	5.2 (3.5–8.9)
Aspirin PO	−45	Early	0–10	NA[a]
Morphine SC	0	Late	+20–35	0.6 (0.5–0.7)
PD 117302 SC	−10	Late	+20–35	0.2 (0.1–0.3)
Aspirin PO	−25	Late	+20–35	NA[a]

[a] Not attained

The effect of morphine on late phase flinching in 70–90 g rats was comparable to that obtained in 200–225 g animals [$A_{50} = 0.3$ (0.2–0.4) mg/kg, SC]. As in the late phase, morphine-induced antagonism of early phase flinching was well correlated with dose, $A_{50} = 1.2$ mg/kg, SC (Fig. 3a). Thus, morphine was 1.9 times more potent against late phase flinching than it was against the acute phase of the response.

A much greater separation between early and late phase effects was observed with the kappa-selective

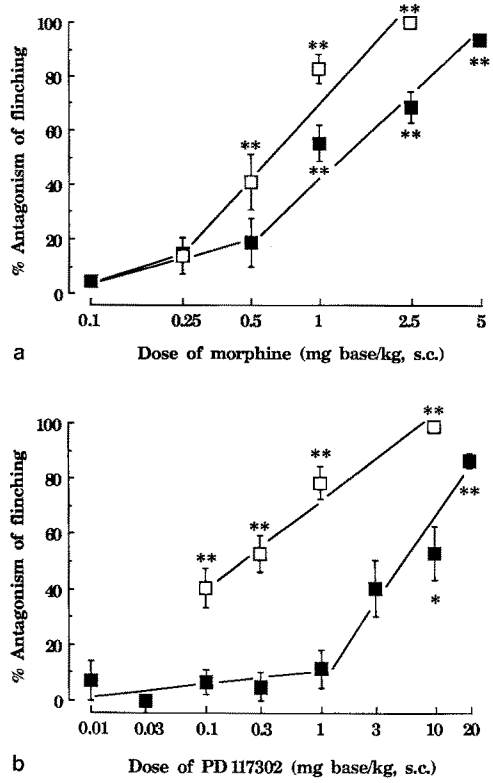

Fig. 3a, b Dose-response relation for antagonism of early (0–10 min) and late (20–35 min) phase formalin-induced flinching in the 70–90 g rat by morphine and PD 117302. **a** Morphine or saline ($n = 8$ or 12) was administered SC 20 min before (early phase, $n = 5$–10) or immediately prior to (late phase, $n = 6$–8) injection of 5% formalin (50 µl, SC) under the dorsal surface of the right hind paw. **b** PD 117302 or saline ($n = 8$) was administered SC 30 min (early phase, $n = 6$–8) or 10 min before (late phase, $n = 10$, except 10 mg/kg where $n = 4$) injection of formalin. Each point is the mean % antagonism of formalin control ± SE. *Asterisks* indicate significant difference from formalin + saline (* $P < 0.05$, ** $P < 0.01$, ANOVA followed by Dunnett's *t* test). ■ Early phase; □ late phase

opioid agonist, PD 117302 (Fig. 3b) which, from parallel line assay, was 27 times more potent against late as opposed to early phase flinching.

In the 50° C tail-dip test, PD 117302 (30 mg/kg, SC) did not increase tail withdrawal latencies (5.3 ± 0.2 and 5.1 ± 0.6 s pre- and post-treatment, respectively). In contrast, morphine increased reaction times at doses shown to be effective in both phases of the formalin test. The A_{50} of morphine in the tail dip test, 1.7 (0.9–4.3) mg/kg, SC was similar to that obtained against early phase formalin-induced flinching.

The antinociceptive effect of morphine on flinching behavior, in both phases of the formalin response, was stereoselectively blocked by naloxone. Morphine alone (2 mg/kg, SC) produced 58 ± 5% inhibition of early phase flinching. (+)-Naloxone (1 mg/kg, SC) did not change the level of antinociception (75 ± 6%) significantly. However, combination with (−)-naloxone (1 mg/kg, SC) completely antagonized the antinociceptive action of morphine (4 ± 2%, $P < 0.05$, ANOVA followed by Scheffé's test, $n = 5$). Neither enantiomer of naloxone significantly affected early phase flinching when given alone. In the late phase, morphine alone (1 mg/kg, SC) produced 70 ± 9% inhibition of flinching. As in the early phase, combination with (+)-naloxone did not alter the antagonism of flinching (75 ± 6%) but administration of (−)-naloxone completely prevented the effect of morphine. Since this dose of naloxone (1 mg/kg) itself enhanced flinching, some care is required in interpreting these data (see Discussion). At 0.03 mg/kg, SC, however, (−)-naloxone alone did not affect late phase flinching but still blocked the inhibition of flinching produced by morphine (morphine = 91 ± 3%; morphine + naloxone = 1 ± 8% inhibition of flinching, $P < 0.05$, ANOVA followed by Scheffé's test, $n = 6$).

Aspirin given acutely at 125, 250 and 500 mg/kg, PO produced a non-dose-dependent reduction of flinching (30–40% maximum antagonism, Table 4). The effect was observed in both phases of the response but it was variable and not statistically significant. Acute aspirin did not inhibit edema formation. Multiple dosing with aspirin (125 mg/kg, PO twice daily for 2.5 days) also failed to antagonize the behavioral effects of formalin (Table 4). Paradoxically, the edematous response to formalin was enhanced in animals receiving repeated doses of aspirin but not in rats given a single oral dose of 125 mg/kg aspirin.

Table 4. Comparison of the effect of acute and multiple administration of aspirin on flinching behavior and paw edema produced by formalin (50 µl, 5%) into the right hind paw. Treated rats received aspirin 125 mg/kg, PO and formalin at + 25 min, or aspirin 125 mg/kg, PO twice daily for 2.5 days followed 12 h later by formalin. Control animals received equivalent doses of vehicle plus formalin

Treatment group	n	Mean no. of flinches ± SE		% Increase in paw volume at T = 90
		0–10 min	20–35 min	
Acute aspirin	7	44 ± 4	59 ± 4	45.9 ± 2.1
Control	7	42 ± 4	68 ± 7	46.0 ± 3.6
Multiple aspirin	8	62 ± 5*	75 ± 8	65.6 ± 2.9**,***
Control	7	55 ± 7	87 ± 6	49.3 ± 4.2

* $P < 0.05$, ** $P < 0.01$. Significance was determined with ANOVA followed by Dunnett's t test and indicates difference from acute aspirin

*** Significantly different from vehicle control ($P < 0.01$, ANOVA followed by Dunnett's t test)

In contrast to the resistance shown to aspirin, formalin-induced edema was at least partially sensitive to morphine and, to a lesser extent, PD 117302. Basal volumes were in the range 0.91–1.35 ml (mean ± SE = 1.09 ± 0.02 ml, $n = 35$). Formalin caused a 36.4 ± 3.8% increase in paw volume in control animals ($n = 5$). The dose of morphine required to produce a 50% reduction in edema, relative to controls, was 2.4 (1.1–NA) mg/kg, SC. An A_{50} value was not obtained for PD 117302. The maximum inhibitory effect of this compound appeared to be ~35% at 1 and 10 mg/kg, SC.

Discussion

In the present paper, we describe a standardized version of the rat paw formalin test for evaluating analgesics and report on important differences between acute and tonic pain.

As previously described (Wheeler-Aceto et al. 1990), the time course of formalin-induced spontaneous nociceptive behavior in the rat correlates well with the original behavioral rating scale of Dubuisson and Dennis (1977), and with the electrophysiological findings of Dickenson and Sullivan (1987). In addition, data obtained from mice (Hunskaar et al. 1985; Murray et al. 1988) indicate a similar time course for formalin-induced nociceptive behavior (licking time) in this species. However, the response in guinea pigs is most comparable to that obtained in the cat using a pain rating scale (Dubuisson and Dennis 1977). Injection of formalin into a forepaw of the cat elicits an immediate peak nociceptive response. After subsiding to a lower level, within 5 min of injection, the response persists for approximately 30 min before gradually decreasing towards baseline at around 60 min. Yet another form of the response is obtained in rhesus monkeys (Alreja et al. 1984). In this species, an initial peak in behavior, over the first 5 min post-injection, is followed by several oscillations in pain rating over the subsequent 60 min. We have observed similar oscillations in licking, and to a lesser extent flinching behavior, in the rat when a 1 min observation period is employed (Wheeler-Aceto et al. 1990).

The data from our laboratory and from other groups demonstrate that formalin produces a quantifiable and reproducible nociceptive response in several species. Notwithstanding some variation, a similar temporal pattern is observed in all species. Each exhibits an initial peak response, which may be more characteristically *phasic* in nature. This is followed by either a second/oscillating peak or a plateau in behavior, both of which appear to represent *tonic* nociceptive stimulation. Interestingly, the intensity and time-course of the pain produced by formalin injection in humans coincides very closely with

that defined by behavioral and electrophysiological measures in other species (Dubuisson and Dennis 1977; HWA unpublished observation).

During the course of developing the rat paw formalin test we have made a variety of observations leading to the recommendations which follow. We advocate use of these methodological revisions as a practical, standardized means of employing formalin-induced tonic pain for the evaluation of antinociceptive activity.

Formalin-induced behavior is very reproducible, in qualitative terms, across a range of age/weights. Our original reason for choosing smaller animals was ease of direct intrathecal injection. However, we recommend the use of smaller animals (less than 200 g) in this test because of their generally higher response level. In addition, evidence from binding studies suggests that kappa receptor density is greatest in young (10–14 days) animals and decreases with age (Barr et al. 1986; Allerton et al. 1989). Moreover, there appears to be a good correlation between binding and in vivo studies in immature rats. Activity in the warm water (50° C) tail-dip test was greatest in 10-day-old animals (Barr et al. 1986) and the behavioral effects of kappa agonists in 5–20-day-old rats were markedly different from those obtained in the adult (Jackson and Kitchen 1989). The use of young (26–30 days; 70–90 g) animals in this model may therefore be more appropriate to the study of agents thought to act at kappa receptors.

Also worthy of note in this regard is the demonstration of a tonic nociceptive response in the guinea pig. This species more closely resembles man and monkey, in terms of the distribution of kappa receptors in the CNS, than does the rat (reviewed by Mansour et al. 1988). In addition, kappa receptors in the guinea pig show a pattern of subtype and affinity state distinct from the rat (Zukin et al. 1988). Using a paw pressure test, Hayes et al. (1987) demonstrated that kappa-preferring agonists are significantly more potent in the guinea pig than in the rat. Furthermore, they suggest that the relative potency of a drug in each species may indicate the degree to which it is selective for kappa receptors in vivo. The paw pressure test described is, however, a phasic and subjective endpoint. The prolonged behavioral response of the guinea pig to formalin may thus provide a particularly valid animal model to study the role of kappa receptors in tonic pain states.

Extensive examination of the time course of formalin-induced behaviors, in both vehicle- and drug-treated animals, has revealed that observations over 0–10 min and 20–35 min post-formalin give reliable and reproducible behavioral scores for early and late phases, respectively. Indeed, A_{50}s and 95% confidence limits obtained using a 15 min observation are comparable to those generated using 20–50 min late phase scores. Shorter observation periods give less satisfactory dose-response relationships. We therefore recommend use of the 0–10 or 20–35 min observation periods, in conjunction with appropriate pretreatment time of the agent under test, for the assessment of antinociceptive activity. In most instances, quantitation of the early and late phase of the response necessitates use of separate groups of animals.

In all of the experiments currently reported both flinching and licking behavior were recorded. Some interesting effects on licking behavior were observed and licking was frequently inhibited in a dose-related manner by pretreatment with analgesics. However, for several reasons, our strongest recommendation for this test is that flinching alone be used for the quantitation of antinociceptive activity. From a purely practical point of view we have found flinching preferable to licking. Flinching or shaking of the paw results in a movement of the back and/or hindquarters which is easy to see, even when the paw itself is not visible. Licking, on the other hand, can be difficult to observe depending on the position of the animal.

The very nature of the licking response, that is, a deliberate action which requires the animal to direct its attention to the injected paw, means that it is often influenced by agents which affect various aspects of behavior. Our observations suggest that licking is readily suppressed by compounds which either stimulate or depress locomotor activity and by those which cause various forms of stereotypy. The same is not true of flinching. It is a more spontaneous and robust behavior and is less contaminated by other behavioral influences. Subjective observations lead us to believe that agents may also suppress licking, particularly in the early phase, by altering taste. The extent of the effect is such that when the paw is licked the exudate becomes sufficiently unpalatable to deter further licking.

In addition to overt behavioral effects, the dose-response relationship against licking was generally less impressive than that obtained using flinching as the endpoint. This was especially evident around the 50% level. Most importantly, the observation of paradoxical actions against licking by naloxone, which are uninterpretable in terms of known or predicted clinically useful analgesic efficacy, militates against the use of licking as an endpoint. The (−)-enantiomer of naloxone, when given alone, affected licking to a level which interfered with intrepretation of morphine antagonism studies. In both phases of the response, licking was inhibited by (−)-naloxone (manuscript in preparation). Thus, in common with the arthritic rat (Kayser and Guilbaud 1981; Kayser et al. 1988) and sciatic nerve ligation (Attal et al. 1989) models of chronic pain, where low doses of naloxone have a paradoxical antinociceptive effect, inhibition of licking by (−)-naloxone ascribes activity to this drug when it is not generally accepted to be analgesic. In contrast, (+)-naloxone had no effect on either phase of formalin-induced behavior.

It has been demonstrated electrophysiologically (Lombard and Besson 1989), behaviorally (Kayser and Guilbaud 1981; Millan et al. 1987) and biochemically (Cesselin et al. 1980; Höllt et al. 1987; Millan et al. 1987) in the arthritic rat that tonic activity of endogenous spinal opioid systems is increased and that naloxone both facilitates the transmission of nociceptive information and causes hyperalgesia. Similar effects have been observed in response to acute peripheral inflammation (Iadarola et al. 1988). Thus, as might be predicted if endogenous opioids are also involved in modulation of

the tonic nociceptive action of formalin, at doses greater than 0.03 mg/kg, SC, (−)-naloxone produced a significant *enhancement* of late phase flinching but did not influence early phase flinching.

In common with our own observations on licking, Vaccarino et al. (1988) demonstrated a significant dose-dependent inhibition of this formalin-induced response by naloxone in BALB/c mice but not C57BL/6 or CD1 mice. It should be noted that, in mice, licking is the only readily quantifiable behavioral response following formalin injection. Similar findings were obtained using naltrexone (Vaccarino et al. 1989) in BALB/c animals. The same group could demonstrate no effect of naloxone on tail-withdrawal latency in this strain of mouse but were able to antagonize the effect of morphine in the tail-flick test. In contrast, while low doses of naloxone attenuated the effect of morphine in the formalin test, high doses potentiated the inhibition of licking produced by morphine. These findings were interpreted as a demonstration of dose-related antinociception by naloxone. However, in view of our findings on flinching we urge caution in the interpretation of licking data from the formalin test strictly in terms of pain and analgesia. North (1978) reported the ability of naloxone (in a single high dose, 10 mg/kg, SC) to reverse morphine analgesia in the rat formalin test but failed to demonstrate an effect of naloxone per se. The apparent discrepancy between this study and our own may be explained in terms of dose. We have found that the effect of naloxone is biphasic. Both the inhibition of licking and enhancement of formalin-induced flinching observed with naloxone are reduced at doses greater than 3 mg/kg, SC.

The spontaneous rhythmicity of the flinching response suggests that this behavior might (like the tail-flick) be a bulbo-spinal reflex. Our spinal transection studies were performed to determine whether part, or all, of this response is spinally processed. Late phase flinching was completely abolished by transection (as alluded to by Ryan et al. 1985), suggesting that the tonic nociceptive signal is supraspinally processed with no involvement of spinal reflex arcs. In contrast, although early phase flinching was significantly attenuated in transected animals, a weak foot withdrawal reflex (up to 40% of the control response) was still observed. In the same animals, the tail withdrawal reflex was significantly enhanced. Although hyper-reflexia was apparent in the tail-dip test at 48 h post-transection, the short post-surgical interval means that presence of spinal shock in these animals cannot be ruled out. It is possible, therefore, that after longer recovery periods an enhancement of the reflex component of early phase flinching would be observed in transected rats. Taken together, these findings suggest that: i) tail withdrawal is spinally processed; the supraspinal component is a modulatory one, removal of which augments the response; ii) early phase flinching has both spinal and supraspinal components involved in its generation; removal of the latter significantly attenuates the response; and iii) late phase flinching is exclusively supraspinally processed; disconnection of spinal and supraspinal components abolishes the response. The range in relative contribution of spinal and supraspinal pathways to the generation of each of these end-points may be of use in defining any physiological and anatomical differences between acute and tonic pain signals.

Presence of formalin did not influence the tail withdrawal reflex, in either transected or sham-operated animals. This may be a consequence of transmission of these two nociceptive stimuli via independent pathways, or utilization of different mechanisms within a given pathway. Such contentions are supported by the observation that lesions of the nucleus raphe magnus, caudal periaqueductal grey (Abbott et al. 1982) and dorsolateral funiculus (Ryan et al. 1985) abolish the antinociceptive effect of morphine in the tail-flick test but fail to attenuate antinociception in the formalin test.

Using the tail withdrawal test and both phases of the formalin response, we have obtained a spectrum of activity with the kappa agonist PD 117302. Although inactive in the tail withdrawal test, PD 117302 shows impressive potency and efficacy against late phase flinching, with intermediate activity against the early phase of the response. Morphine, on the other hand, is essentially equipotent against all three end-points. We believe this striking difference in profile may be related to the intensity of the stimulus, in terms of the frequency of firing produced in the primary dorsal horn neuron, associated with each end-point.

Work from several groups has suggested that kappa agonists are significantly more effective against noxious mechanical and chemical stimuli than they are against noxious heat (Tyers 1980; Upton et al. 1982; Schmauss et al. 1983; Schmauss and Yaksh 1983; Millan 1986; Schmauss 1987). These differences in efficacy have been attributed to a modality specific action of kappa agonists. However, more recent behavioral (Millan et al. 1988b; Shaw et al. 1988; Millan 1989) and electrophysiological (Headley and Parsons 1989; Parsons and Headley 1989) studies suggest that when the intensity of different modes of noxious stimuli are matched, any apparent selectivity is lost.

It has been demonstrated (Dickenson and Sullivan 1987) that the phasic peak of dorsal horn neuron firing in response to formalin is of significantly higher frequency than the tonic excitatory response (mean maximum 22 and 12 spikes/s, respectively). The former may be most comparable to the evoked neuronal firing produced in the ventral root by moderate pinch and mild thermal stimuli (Parsons and Headley 1989). On the other hand, the 50° C thermal stimulus used in our tail-withdrawal test is more likely to evoke a more intense level of firing (> 30 spikes/s, Parsons and Headley 1989) in both dorsal and ventral root neurons. Thus, the spectrum of activity observed with PD 117302 in the present study appears to be consistent with the notion that intensity of stimulus and not modality is paramount in determining the analgesic profile of kappa agonists. Moreover, in view of the effect of transection on each of the end-points presently described, we speculate that such a hypothesis can be extended to cover processing of nociceptive information at spinal and supraspinal levels. It seems possible that only higher intensity stimuli (like thermal tail-flick and early phase formalin) are able to operate spinal reflex arcs. More intriguing, however, is the prospect that the frequency of firing elicited by a stimulus is responsible for

differentially activating distinct spinal and/or supraspinal pain-signalling systems, similar to those long advocated by Melzack and colleagues (Dennis and Melzack 1979; Abbott et al. 1982; Cohen and Melzack 1985; for recent review see Melzack 1990). Since frequency of firing evoked in dorsal horn neurons involved in the transmission of nociceptive information is likely to be a function of: i) the modality of the stimulus, ii) the intensity of the stimulus, iii) the duration of the stimulus and iv) the degree of tissue damage sustained and consequent release of inflammatory and algogenic substances, a concept of this nature could have important implications for the study of pain and the development of new analgesics.

Efforts are currently underway in our laboratory to rank different classes of analgesic against all three stimulus levels. The profiles obtained will be compared with known clinical data or used to make predictions regarding clinical efficacy. Included in these studies are high efficacy non-steroidal anti-inflammatory drugs. In contrast to the findings of Hunskaar et al. (1986) and Hunskaar and Hole (1987) with mice, we were unable to demonstrate consistent antinociception with aspirin in either phase of the formalin test. Our observation that repeated administration of aspirin enhanced formalin-induced edema suggests that a lipoxygenase product may, at least in part, be responsible for edema production. Inhibition of cyclooxygenase by aspirin could lead to increased flux of arachidonate through the lipoxygenase pathway. Although a product of lipoxygenase may be involved in edema formation, it is unlikely to be responsible for pain production, since the behavioral response to nociception was unchanged after repeated aspirin. In contrast, edema was partially reduced by morphine (50%) and PD 117302 (35%), both of which attenuate pain-related behavior. Collectively, these results point to the involvement of a neurogenic component in edema formation.

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