



## THE MAJOR ROLE OF PERIPHERAL RELEASE OF HISTAMINE AND 5-HYDROXYTRYPTAMINE IN FORMALIN-INDUCED NOCICEPTION

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**Abstract**—Formalin injected subcutaneously into the paw is a widely used model of pain. This procedure evokes a short-lasting period of flinching (phase 1) and a long-lasting period of intense flinching (phase 2) following a very short period of quiescence. Phase 2 has been extensively used to support the involvement of central (spinal cord) sensitization in inflammatory hyperalgesia. The present study evaluated the contribution of stimulation of peripheral nociceptors by the release of endogenous mediators at the site of lesion. The participation of histamine and 5-hydroxytryptamine was demonstrated by the treatment of the rat hindpaws with selective histamine H1 (pyrilamine and meclizine) and histamine H2 (cimetidine) receptor antagonists or selective 5-hydroxytryptamine<sub>1A</sub> (WAY100,135) and 5-hydroxytryptamine<sub>4/3</sub> (tropisetron) receptor antagonists. The co-administration of pyrilamine or meclizine with formalin (1%) significantly reduced phases 1 and 2, while cimetidine had no effect. Pyrilamine administration during the period of quiescence (10 min after formalin administration) caused strong dose-related inhibition of phase 2. The co-administration of tropisetron with formalin caused a blockade of both phases, while with WAY100,135 caused only inhibition of the phase 2. In contrast, tropisetron administered during the period of quiescence did not cause antinociception. Histamine and 5-hydroxytryptamine receptors could be strongly activated in naïve animals by administration of a mixture of both agonists or compound 48/80 (2 µg/paw) which is known to release both mediators from mast cells. Pretreatment of the paws with a mast cell stabilizer, sodium cromoglycate, significantly reduced the second phase of the formalin injection model.

From these results we suggest that phases 1 and 2 of the formalin test are dependent upon the ongoing afferent input. Furthermore, while histamine H1 participates in both phases, 5-hydroxytryptamine<sub>4/3</sub> participates in phase 1 and 5-hydroxytryptamine<sub>1A</sub> in phase 2. © 2001 IBRO. Published by Elsevier Science Ltd. All rights reserved.

**Key words:** histamine, serotonin, formalin test, hyperalgesia, peripheral inflammatory mediator, nociceptive response.

Introduced by Dubuisson and Dennis<sup>13</sup> in 1977, the formalin test is widely used to evaluate analgesic drugs, being considered an animal model of tonic inflammatory pain.<sup>2,4,42</sup> However, the relative contribution of peripheral and central sensitization mechanisms involved in the nociceptive responses is not fully understood.

Subcutaneous injection of formalin into the rat hind-paw evokes an array of stereotyped behaviours. Among these behaviours, flinching (consisting of an elevation and shrinking back of the injected paw) is a reliable parameter of pain behaviour.<sup>40</sup> The nociceptive response to formalin occurs in a biphasic pattern: there is an initial acute period (phase 1, duration of 7–10 min), and after a short period of remission, phase 2 begins and consists of a longer period (1 h) of sustained activity.<sup>26,39,41,43,44</sup> The initial response was initially attributed to a direct algogenic effect of formalin on the nociceptors<sup>23,34</sup> whereas phase 2 was associated with the release of local endogenous mediators responsible for sensitization of primary and spinal sensory neurons and subsequent activation of the nociceptors. The simple direct activation of primary

sensory fibres by formalin in the first phase is questionable, since bradykinin and some 5-hydroxytryptamine (5-HT) antagonists significantly diminished such responses.<sup>9,12</sup>

Spinal cord sensitization has been emphasized by various authors as a major factor in the induction and maintenance of the second phase of behaviour.<sup>5–8,10,11,45</sup> This sensitization may result from the pre-synaptic release of mediators such as glutamate<sup>5,26,27,46</sup> and substance P.<sup>5,30,37</sup> Consistent with this idea was the demonstration that intrathecal administration of lidocaine,<sup>7,10,49</sup> morphine,<sup>11</sup> *N*-methyl-D-aspartate (NMDA) antagonists<sup>6,16,47</sup> or substance P antagonists<sup>30,32,48</sup> reduced behavioural responses and/or the activity of dorsal horn neurons when administered prior to, but not immediately after, phase 1. However, other authors concluded that persistent input or firing of primary afferent fibres was correlated with phase 2 behaviour activity evoked by formalin.<sup>3,20,29,34,42</sup>

Putative inflammatory mediators such as histamine, serotonin, bradykinin and prostaglandins are also able to induce dose-dependent nociceptive behaviours when injected into the paw.<sup>4,19,35</sup> As noted above, 5-HT has been shown to be involved in the first phase of the formalin test.<sup>12</sup> Histamine and 5-HT are present in mast cells which concomitantly release both amines following inflammatory insult. Although Shibatta *et al.*<sup>39</sup> reported

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Abbreviations: ANOVA, analysis of variance; 5-HT, 5-hydroxytryptamine; NMDA, *N*-methyl-D-aspartate.

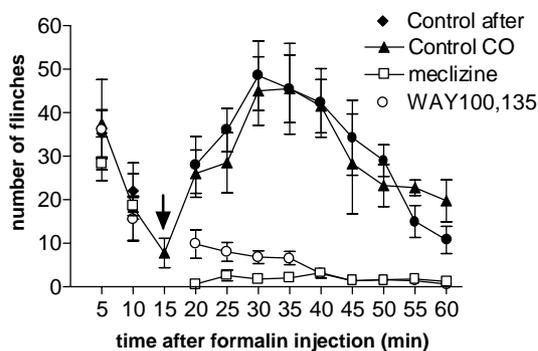


Fig. 1. Effect of post-treatment (15 min after s.c. injections of formalin 1%, arrow) with the histamine H1 receptor antagonist meclizine (400  $\mu\text{g}/\text{paw}$ ) and 5-HT<sub>1A</sub> receptor antagonist WAY100,135 (450  $\mu\text{g}/\text{paw}$ ) on the flinching time-course. Control saline (20  $\mu\text{l}$ ) was co-injected with (full triangles) or injected 15 min after formalin (full dots).

that mast cell depletion inhibits the second phase of the formalin test, the role of histamine in formalin-induced nociception has not been systematically evaluated.

Using histamine H1- and H2-receptor antagonists, we investigated the involvement of the effects of endogenous release of histamine on both phases of the formalin test. Using a parallel approach we examined the role of 5-HT<sub>1A</sub> and 5-HT<sub>3/4</sub> receptor in formalin behaviour using specific receptor antagonists. Finally, the potential role of mast cells was examined by determining the effect of a combination of histamine and 5-HT, the effect of compound 48/80 and the effect of pretreatment with cromolyn.

## EXPERIMENTAL PROCEDURES

### Animals

This study was carried out using male Wistar rats (University of São Paulo) weighing between 120 and 160 g. The animals were housed in groups of six in an animal care facility and maintained on a 12-h light/dark cycle with rat chow and water available *ad libitum*. On the day of testing, rats were removed from the animal care facility and taken to the testing area at least 1 h before testing. Each experiment used four or six rats per group. All experimental work involving animals conformed to the IASP guidelines on the use of animals in pain research. Rats were used once only. All efforts were made to minimize the number of animals used and their suffering.

### Formalin test

Rats were placed in an open Plexiglas observation chamber for 30 min to accommodate to their surroundings, then removed for formalin administration. Rats were gently restrained while the dorsum of the hindpaw was subcutaneously administered with 50  $\mu\text{l}$  of formalin 1% (1:100 dilution of stock formalin solution, 37% formaldehyde in 0.9% saline) using a 30 gauge needle. Following injection, the rat was returned to the observation chamber for a 60-min observation period. A mirror was placed behind the chamber to enable unhindered observation of the formalin-injected paw. The recording time was divided into 12 blocks of 5 min and a pain score was determined for each block by measuring the number of lifts or flinches of the affected limb during the observation time. Such behaviour could vary from a simple lift of the paw (not associated with locomotion) to a vigorous shaking of the limb, or it could be a rippling of the back

muscle associated with limb movement. Lifts or flinches were discrete and easily quantifiable.

### Materials

The following drugs were used: selective H1-receptor antagonists pyrilamine maleate salt (50, 100 and 400  $\mu\text{g}$ ), meclizine dihydrochloride (25, 100 and 400  $\mu\text{g}$ ) and the mast cell depletor, compound 48/80 (2  $\mu\text{g}$ ), were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The selective histamine H2-receptor antagonist cimetidine (25, 100 and 400  $\mu\text{g}$ ), 5-HT<sub>3/4</sub> receptor antagonist tropisetron hydrochloride<sup>22</sup> (50, 150 and 450  $\mu\text{g}$ ), histamine dihydrochloride (25, 100 and 250  $\mu\text{g}$ ) and 5-hydroxytryptamine (25, 50 and 250  $\mu\text{g}$ ) were obtained from Research Biochemicals (Natick, MA, USA). The selective 5-HT<sub>1A</sub> receptor antagonist (+) WAY100,135 (25, 100 and 400  $\mu\text{g}$ ) was obtained from Wyeth Research, and the membrane mast cell stabilizer sodium cromoglycate (cromolyn; 800  $\mu\text{g}$ ) was purchased from Rhodia Farmacos (SP, Brazil). All drugs were diluted in 0.9% saline solution, except for meclizine, which was diluted in cremophor.

### Testing procedures

All the receptor antagonists used were co-administered with formalin or administered 10 min after the formalin injection. Cromolyn was administered 15 min before the formalin injection. These drugs were administered in a volume of 20  $\mu\text{l}$ . The same volume of saline or cremophor (meclizine vehicle) was used as control. The formalin-evoked nociceptive flinching behaviour was evaluated immediately after the formalin injection for 60 min. To rule out the possibility of a systemic action, the highest doses of the drugs were administered (s.c.) into the contralateral paw to formalin injection, and the nociceptive response was evaluated.

In another set of experiments, the nociceptive flinching behaviour was evaluated immediately after the administration (s.c.) of histamine (50 and 250  $\mu\text{g}$ ) or 5-HT (50 and 250  $\mu\text{g}$ ), compound 48/80 (2  $\mu\text{g}$ ) or the combination of histamine (25  $\mu\text{g}$ ) with 5-HT (25  $\mu\text{g}$ ) in a volume of 50  $\mu\text{l}$ , for 30 min following injection. The same volume of saline or a single dose of histamine (50  $\mu\text{g}$ ) was used as control.

The formalin-evoked nociceptive flinching behaviour was divided into phase 1 (0–10 min) and phase 2 (15–60 min) as previously described.<sup>13,34,39,44</sup> Behaviour during the first 15 min was not considered in the groups that underwent post-phase 1 injection.

### Statistical analysis

Data collected were analysed using one-way analysis of variance (ANOVA). Only the total number of flinches in each period analysed was used for the statistical tests. Multiple post-hoc comparisons were performed using Tukey's test. Statistical significance was accepted at the 5% level ( $P < 0.05$ ). Results are presented as mean ( $\pm$ S.E.M.).

## RESULTS

### Time-course of flinching behaviour induced by formalin and blockade of the phase 2 by H1 or 5-HT<sub>1A</sub> receptor antagonists given after phase 1

The time-course of nociceptive behaviour (flinches, phases 1 and 2) after formalin injection is shown in Fig. 1. Saline either co-administered with formalin or administered after phase 1 did not change the time-course of flinching behaviour. This figure also shows the time-course of the inhibition of the nociceptive behaviour observed when the histamine H1 receptor antagonist meclizine or the 5-HT<sub>1A</sub> receptor antagonist WAY 100,135 was administered locally just after the end of phase 1 (15 min).

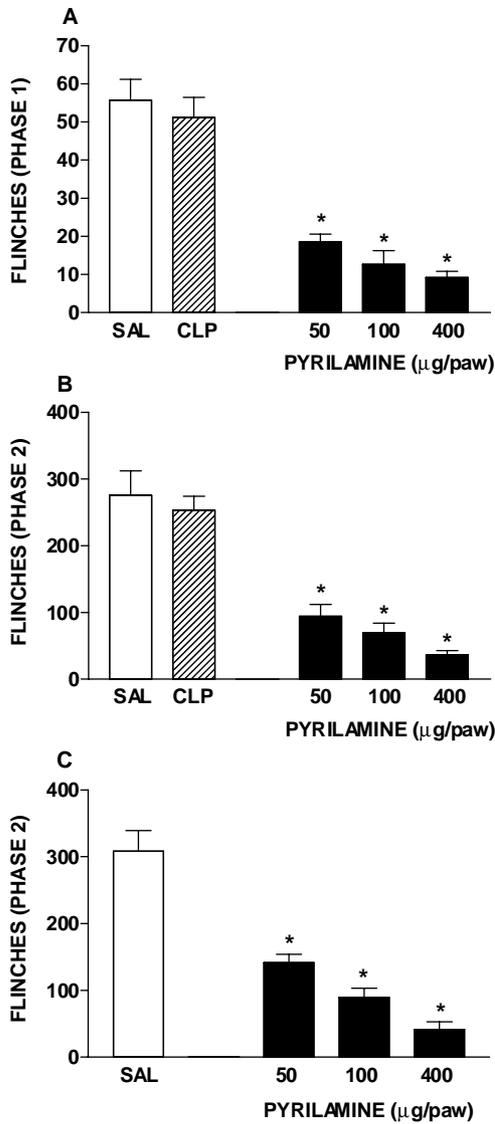


Fig. 2. Dose-related blockade of formalin 1% induced flinches by co-treatment (A and B) or post-treatment (C) with the histamine H1 receptor antagonist pyrilamine. Pylarimine (50, 100 and 400 µg) or saline (20 µl) was injected into the ipsilateral paw. A control injection of 400 µg/paw was given into the contralateral paw (CLP). \*Significant difference ( $P < 0.001$ , ANOVA) from saline (SAL). Values are means  $\pm$  S.E.M. (six animals per group).

#### Effects of the selective histamine H1 and H2 receptor antagonists on formalin-evoked nociceptive flinching behaviour

Figures 2–6 show the effects of the co-administration of the antagonists and formalin on phase 1 (panel A) and phase 2 (panel B) or phase 2 when the antagonists were given after phase 1 (panel C)

The co-administration of the H1-receptor antagonists significantly inhibited in a dose-response manner the formalin-evoked nociceptive flinching behaviour ( $P < 0.001$ ) in both phases (Figs 2A, B, 3A, B). At a dose of 400 µg, pyrilamine as well as meclizine reduced the formalin-evoked nociceptive flinching behaviour (95% and 87% inhibition, respectively). In contrast, the

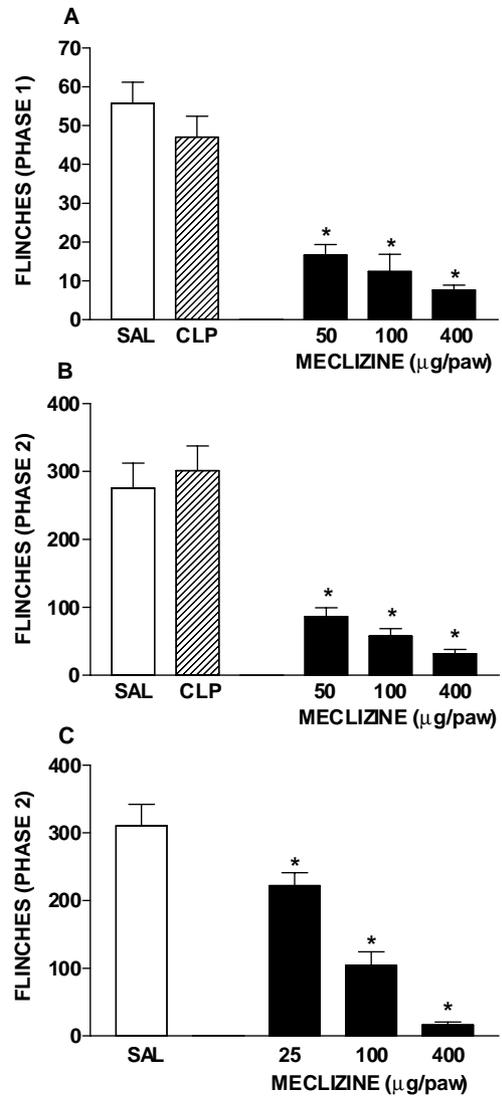


Fig. 3. Dose-related blockade of formalin 1% induced flinches by co-treatment (A, B) or post-treatment (C) with the histamine H2 receptor antagonist meclizine. Meclizine (50, 100 and 400 µg) or saline (20 µl) were injected into the ipsilateral paw. A control injection of 400 µg/paw was given into the contralateral paw (CLP). \*Significant difference ( $P < 0.001$ , ANOVA) from saline (SAL). Values are means  $\pm$  S.E.M. (six animals per group).

co-administration of the selective histamine H2-receptor antagonist had little effect upon formalin-induced behaviours, only reducing such behaviours at a dose of 400 µg ( $P < 0.05$ , Fig. 4A, B). The post-administration (Figs 2C, 3C) of the H1-receptor antagonists, pyrilamine and meclizine, significantly inhibited phase 2 in a dose-response manner but the histamine H2-receptor antagonist cimetidine had no effect (Fig. 4C).

#### Effects of the selective 5-HT<sub>1A</sub> and 5-HT<sub>3/4</sub> receptor antagonists on the second phase of formalin-evoked nociceptive flinching behaviour

The role of endogenous 5-HT in phase 2 of flinching behaviour in response to subcutaneous injection of formalin was investigated by administration of

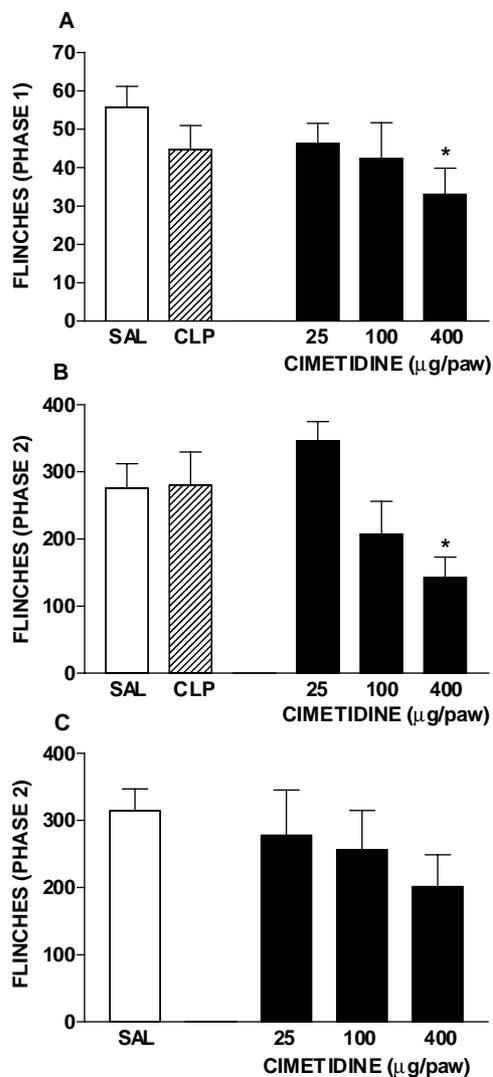


Fig. 4. Dose-related blockade of formalin 1% induced flinches by co-treatment (A, B) or post-treatment (C) with the histamine H<sub>2</sub> receptor antagonist cimetidine. Cimetidine (25, 100 and 400 µg) or saline (20 µl) was injected into the ipsilateral paw. A control injection of 400 µg/paw was given into the contralateral paw (CLP). \*Significant difference ( $P < 0.05$ , ANOVA) from saline (SAL). Values are means  $\pm$  S.E.M. (six animals per group).

(+)WAY100,135 (selective 5-HT<sub>1A</sub> antagonist) or tropisetron (selective 5-HT<sub>3/4</sub> antagonist) after phase 1 of formalin 1% injection. The co- and post-administration of (+) WAY100,135 with formalin had an intense effect in phase 2 (Fig. 5B, C), while the co-administration had little effect on phase 1 (panel A). The co-administration only reduced the phase 1 behaviours at a dose of 450 µg ( $P < 0.05$ ).

The co-administration of tropisetron with formalin significantly reduced both phases of flinching behaviour (Fig. 6A, B) but had no effect when administered after phase 1 (Fig. 6C).

None of the highest doses of histamine or 5-HT receptor antagonists administered (s.c.) into the contralateral paw reduced the nociceptive flinching behaviour, ruling out a possible systemic action (Figs 2–6). In order to test whether their effect was due to the possible local

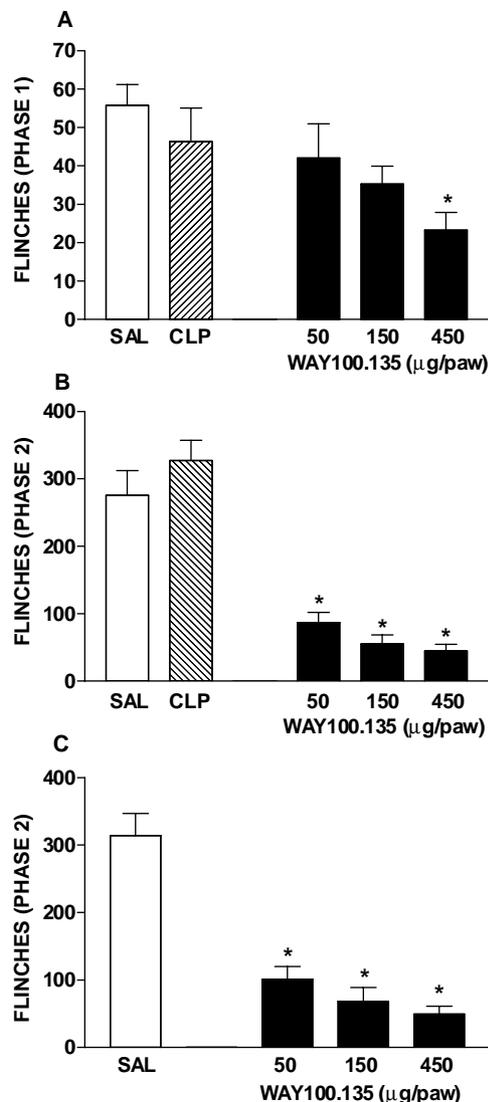


Fig. 5. Dose-related blockade of formalin 1% induced flinches by co-treatment (A, B) or post-treatment (C) with 5-HT<sub>1A</sub> receptor antagonist WAY100,135. WAY100,135 (50, 150 and 450 µg) or saline (20 µl) was injected into the ipsilateral paw. A control injection of 450 µg/paw was given into the contralateral paw (CLP). \*Significant difference ( $P < 0.001$ , ANOVA) from saline (SAL). Values are means  $\pm$  S.E.M. (six animals per group).

anaesthetic activity demonstrated by some antihistaminic agents,<sup>36</sup> additional experiments were performed. Thus, the administration of 400 (µg/paw) s.c. of pyrilamine affected neither the pain threshold in the plantar test<sup>17</sup> ( $10.66 \pm 1.56$  s in basal conditions versus  $10.39 \pm 0.57$  s, 15 min after subcutaneous pyrilamine injection) nor the mechanical hyperalgesia<sup>14</sup> measured 3 h after injection of 100 µg of carrageenin ( $16.13 \pm 1.56$  s compared with  $16.05 \pm 1.53$  s, 15 min after pyrilamine injection).

#### Nociceptive flinching behaviour evoked by serotonin and histamine

The administration (s.c.) of 5-HT (50 µg, 250 µg), histamine (50 µg, 250 µg) or the combination of 5-HT

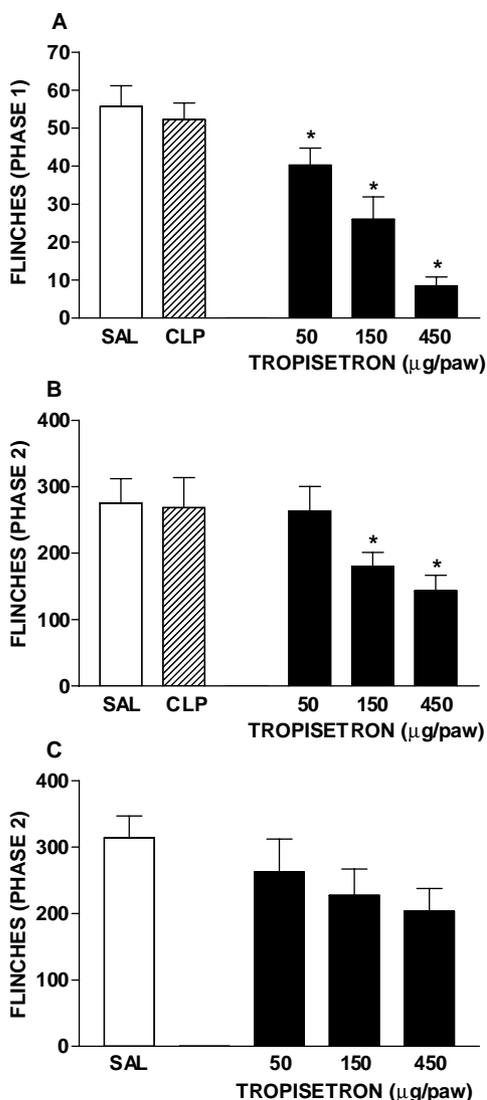


Fig. 6. Dose-related blockade of formalin 1% induced flinches by co-treatment (A, B) or post-treatment (C) with 5-HT<sub>3/4</sub> receptor antagonist tropisetron. Tropisetron (50, 150 and 450 µg) or saline (20 µl) was injected into the ipsilateral paw. A control injection of 450 µg/paw was given into the contralateral paw (CLP). \*Significant difference ( $P < 0.05$ , ANOVA) from saline (SAL). Values are means  $\pm$  S.E.M. (six animals per group).

(25 µg) and histamine (25 µg) induced nociceptive flinching behaviour of the injected paw during the 30-min period of observation (Fig. 7). Administration of the combination of 5-HT (25 µg) and histamine (25 µg) induced a significantly greater number of nociceptive flinches compared to the administration of 5-HT or histamine alone ( $P < 0.001$ ). 5-HT (50 µg) induced a significantly greater nociceptive flinching behaviour compared to histamine (250 µg;  $P < 0.05$ ).

#### Effect of cromolyn and compound 48/80 on nociceptive flinching behaviour

Pretreatment of the paw with cromolyn 4% (s.c., 20 µl) for 15 min before the formalin injection reduced the formalin-evoked nociceptive flinching behaviour in

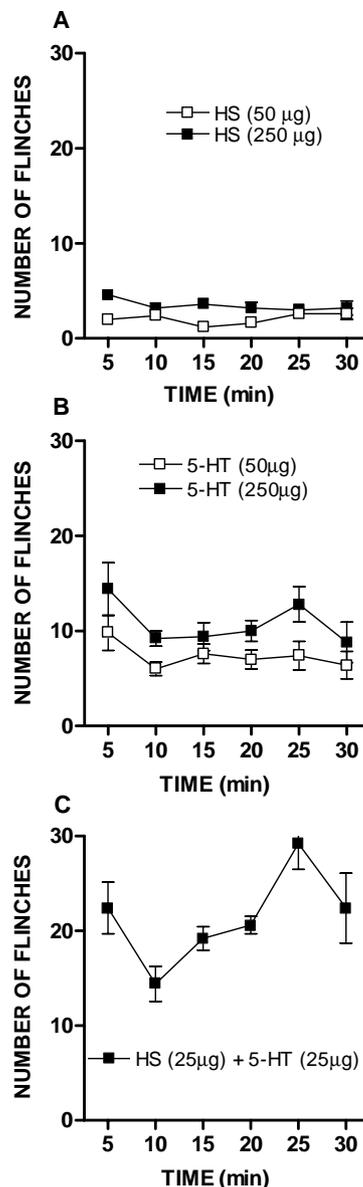


Fig. 7. Time-course of flinching behaviour occurring during a 30-min test period after subcutaneous injection of (A) 50 µg and 250 µg of 5-hydroxytryptamine (5-HT), (B) 50 µl and 250 µg of histamine (HS) and (C) combination of 25 µg of HS and 25 µg of 5-HT (HS + 5-HT). The total number of flinches in C and D differs significantly ( $P < 0.05$  ANOVA) from A and B (both doses). Values are means  $\pm$  S.E.M. (five animals per group).

phases 1 and 2, by 40% and 70%, respectively. However, only phase 2 was significantly reduced compared to saline ( $P < 0.05$ , Fig. 8A). When administered (s.c.) into the contralateral paw, cromolyn failed to reduce the nociceptive flinching behaviour, ruling out a possible systemic action. In contrast to formalin, the nociceptive flinching behaviour induced by compound 48/80 was continuous and not biphasic (Fig. 8B).

#### DISCUSSION

In the present investigation, flinches were used to quantify formalin-induced behaviours since they correlate

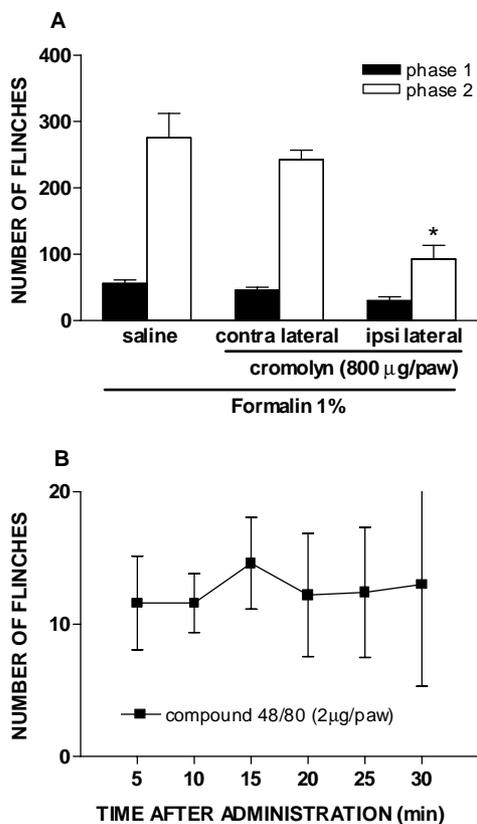


Fig. 8. Effects of pretreatments with sodium cromoglycate (cromolyn) in formalin nociception (A) and induction of flinching by sub-cutaneous injection compound 48/80 in the rat paw (B). The cromolyn (800 µg/paw) or saline (20 µl) was administered 15 min before the administration of formalin. \*Significant difference from saline and contralateral paw ( $P < 0.05$ , ANOVA). Values are means  $\pm$  S.E.M (six animals per group).

well with the formalin-evoked cardiovascular responses and provide a reliable correlation of pain in the awake, freely moving rat.<sup>40</sup> The flinch frequency is simple to measure and one of the highest scoring amongst the various stereotyped behaviours observed in the formalin test.<sup>41</sup>

It has been demonstrated that mepyramine co-administrated with formalin inhibits both phases of nociceptive behaviour.<sup>38</sup> The co-administration of pyrilamine, a selective H1-receptor antagonist, with formalin resulted in a significant and dose-related inhibition of both phases of flinching. In contrast, cimetidine, a selective H2-receptor antagonist, only produced antinociception at the highest dose tested. These results suggest that histamine H1 receptors are relatively more important than H2 receptors in formalin-induced nociceptive behaviour. Hagermark *et al.*<sup>15</sup> demonstrated that in human skin histamine H2-receptor antagonists are less effective than H1-receptor antagonists in reducing the cutaneous itch induced by intradermal injections of histamine. However, it is also possible that the weak inhibitory effects observed after administration of the histamine H2 antagonist are due to non-specific effects of high doses of cimetidine.

The observed local antinociceptive effect of the

antihistaminics could potentially be due to a central analgesic or local anaesthetic effect. Several authors have described a central analgesic effects of the antihistaminic agents.<sup>21,25,36</sup> Systemic injections of pyrilamine cause analgesia in the hot-plate test (used to test centrally acting drugs) as well as in the paw pressure test, which detect peripheral and central acting analgesics.<sup>28</sup> In the present study, a systemic action of histamine H1 and H2-receptor antagonists was ruled out by the lack of effectiveness when drugs were administered in the contralateral paw. In addition, these actions do not appear to result from a local anaesthetic effect,<sup>36</sup> as injection of an effective intraplantar paw dose of pyrilamine did not affect the pain threshold in either the thermal test or the rat paw pressure test of both normal (saline control) and carrageenin-inflamed paws. Thus, the inhibition of the formalin-evoked nociceptive flinching behaviour by antihistaminic agents seems to be associated with the release of peripheral endogenous histamine.

There is an apparent contradiction in our observation that the histamine H1 receptor antagonist almost abolished formalin-induced nociception, and that the s.c. injection of histamine into the paw induced only a small number of flinches. This result agrees with the weak histamine nociceptive behavioural responses previously observed by Hong and Abbott.<sup>19</sup> Thus, in spite of the observation that histamine injected s.c. induces pain,<sup>41</sup> it is not possible to explain the high number of flinches induced by formalin only by the release of endogenous histamine.

Serotonin has been suggested to be an important peripheral nociceptive mediator since its s.c. administration caused intense flinching and licking behaviours.<sup>4,19</sup> Hong and Abbott<sup>20</sup> have shown previously, and we show here, that the association of serotonin with histamine causes a synergism in their effects. In fact, pretreatment of the paws with a small dose of serotonin changed the plateau response of histamine to the higher level observed with high doses of serotonin.

It is known that 5-HT and histamine are synthesized and stored in different granules in resident mast cells.<sup>18</sup> The well-known mast cell secretagogue compound 48/80<sup>24</sup> produced a high number of flinches when given s.c. into the paws (Fig. 8). Thus, it is plausible that formalin is simultaneously releasing both mediators from the mast cells. Supporting this suggestion, we have shown that the injection of a single high dose of sodium cromoglycate, which inhibits mast cell degranulation,<sup>33</sup> significantly reduced formalin-induced responses. In humans, topical sodium cromoglycate has been shown to be effective in the treatment of moderate to severe skin dermatitis.<sup>31</sup>

The second phase of the formalin test has been largely used as a behavioural model of injury-induced central sensitization.<sup>6,8,10,11,47</sup> Some studies, however, suggest that afferent input during the first phase is not sufficient to maintain second phase central sensitization in the dorsal horn.<sup>3,20,29,34,40</sup> In line with this idea, the present data show that the s.c. administration of the H1-receptor antagonists meclizine or pyrilamine as well as the 5-HT<sub>1A</sub> receptor antagonist WAY100,135 given 15 min after the formalin injection significantly

reduced the formalin-evoked nociceptive flinching behaviour in a dose-related manner. Thus, afferent stimulation by endogenous mediators is necessary for phase 2 flinch responses. This does not imply, though, that phase 1 nociceptor stimulation does not contribute to the enhancement of phase 2 responses by either peripheral or central sensitization. In fact, we have shown that cimetidine which inhibited phase 1 but had no effect when given post-phase 1 caused a reduction in phase 2 nociceptive behaviour. The same occurred with the 5-HT<sub>3/4</sub> antagonist, tropisetron, which we demonstrated here not to affect phase 2 when given after formalin, but instead, blocked both phases when given together with formalin.<sup>12</sup> It seems that participation of 5-HT<sub>2A</sub> receptor in nociception induced by formalin depends on the type of behaviour used for quantification. Preliminary experiments with co-administration of ketanserin (400 µg/paw, data not shown) with formalin confirmed the lack of effect of this 5-HT<sub>2A</sub> antagonist when flinching behaviour

was used for nociception quantification.<sup>12</sup> This observation contrasts with that made using lifting and licking behaviours for nociceptive quantification in the formalin test. In such experiments it was observed that pretreatment of the paws with several 5-HT<sub>2A</sub> antagonists had little or no effect in phase 1, but strongly inhibited the phase 2 behaviours.<sup>1</sup> This result also suggested the necessity of the stimulation of 5-HT for eliciting phase 2 behaviour.

#### CONCLUSIONS

The present results demonstrate that peripheral input is required for the nociceptive behaviour of phase 2, which is associated with a simultaneous endogenous release of histamine and serotonin.

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