Anti-inflammatory activity of bee venom peptide 401 (mast cell degranulating peptide) and compound 48/80 results from mast cell degranulation *in vivo*

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1 The relationship between the anti-inflammatory activity of the bee venom peptide 401 in the carrageenin-induced oedema of the rat hind paw and its mast cell degranulating activity has been re-investigated.

2 Mast cell degranulation caused by compound 48/80 (10 mg kg⁻¹) or by allergen challenge in rats sensitized to *Nippostrongylus brasiliensis* also suppressed rat hind paw oedema in the same test.

3 The anti-inflammatory activities of peptide 401 and compound 48/80 were partially suppressed by pretreatment of rats with mepyramine and methysergide, at doses (2.5 mg kg⁻¹) that completely suppressed skin reactions to these mast cell-derived amines. Pretreatment of rats with compound 48/80 also suppressed the apparent anti-inflammatory actions of peptide 401 and of compound 48/80.

4 Injection of peptide 401 together with carrageenin increased the inflammatory response in the rat hind paw.

5 The anti-inflammatory activity of peptide 401 and of compound 48/80 in the carrageenin-induced swelling of the rat hind paw arises from mast cell degranulation *in vivo*.

Introduction

Peptide 401 (MCD peptide) contains 22 residues and occurs in the venom of the common European honey bee to the extent of about 2% by weight (Gauldie *et al.*, 1976; Breithaupt & Habermann, 1968). It has powerful anti-inflammatory activity (at doses as low as 0.1 mg kg⁻¹) in a variety of animal models, i.e., hind paw oedema in the rat induced by carrageenin or turpentine, adjuvant arthritis in the rat, and increased skin permeability in the rat resulting from subcutaneous injection of bradykinin, prostaglandin E₁, kallikrein, histamine and 5-hydroxytryptamine (5-HT) (Billingham *et al.*, 1973; Hanson *et al.*, 1974). It has a powerful degranulating effect on mast cells *in vitro* and this, of course, involves the release of histamine and other pharmacologically active agents. It was originally reported (Billingham *et al.*, 1973; Hanson *et al.*, 1974) that the anti-inflammatory activity of the peptide was distinct from and did not depend on its action on mast cells. On this basis, it was suggested (Billingham *et al.*, 1973) that the peptide might be a useful therapeutic agent in some arthritic conditions. This suggestion is perhaps an echo of the long-held belief that bee venom has some curative effects in rheumatoid arthritis (Beck, 1935).

We made a number of chemically defined derivatives of the peptide in the hope of decreasing its ability to degranulate mast cells while retaining the anti-inflammatory activity. We found, however, that the two activities changed roughly in parallel (Banks *et al.*, 1980; Dempsey, 1981). We also failed to demonstrate any direct effect of peptide 401 on rat polymorphonuclear leukocytes which might mediate anti-inflammatory activity (Dempsey *et al.*, 1985).

These results led us to re-examine the pharmacology of the anti-inflammatory activity of peptide 401 in the rat paw oedema test. We find that the conclusions drawn by previous workers were incorrect. The peptide is not a true anti-inflammatory agent but exerts its activity through degranulation of mast cells, i.e., it is a counter-irritant. Preliminary accounts of this work have been published (Banks *et al.*, 1980; 1983).

Methods

Carrageenin hind paw oedema

Male Sprague-Dawley rats weighing 125 to 150 g were randomly caged in groups of five and given food and water *ad libitum*. The volumes of both hind paws of each rat were measured with a calibrated volume-differential meter (Ugo Basile, Milan). Each rat was injected, subplantar, with 0.1 ml of a 1% solution of carrageenin in physiological saline, unless otherwise stated. Rats receiving drug treatments were injected subcutaneously with 0.1 ml of the test solution immediately after the carrageenin injection. The control groups were injected subcutaneously with the same volume of 0.9% saline. After injections, the volumes of the rat hind paws were measured over a period of 5 h as indicated in the figures. The effects of antagonists to histamine (H₁, mepyramine; H₂, cimetidine), or 5-HT (methysergide) were determined by injecting the antagonist subcutaneously 45 min before the carrageenin injection.

The increase in paw volume was calculated in the following way. The meter reading was first converted to a volume following calibration and, at each time, *t*, the increase in volume $V_t - V_0$ (where V_t is the volume at time *t* and V_0 is the initial volume before treatment) was determined. Mean V_t is the sum of all V_t in a group divided by *n*, the number of animals in the group. Volumes of both the hind paw injected with carrageenin (the right paw, R) and the uninjected hind paw (L) were measured. Significance values were calculated by Student's *t* test.

In some experiments, peptide 401 was injected with carrageenin in a total volume of saline of 0.1 ml.

Pre-depletion of mast cells

Rats were depleted of mast cell-derived mediators according to the regimen of Di Rosa *et al.* (1971). Rats received 0.6 mg kg⁻¹ of compound 48/80 intraperitoneally (0.1% w/v in physiological saline) twice daily for 3 days and 1.2 mg kg⁻¹ twice on the fourth day. The efficacy of the pretreatment was evident from the course of the carrageenin oedema in depleted rats compared with normal animals, swelling in the first hour

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after carrageenin injection being virtually absent in the depleted animals.

Sensitization of rats with the nematode *Nippostrongylus brasiliensis*

Rats were injected sub-cutaneously with third stage larvae of *Nippostrongylus brasiliensis* (L3 1800) in 0.4 ml sterile physiological saline. Three weeks later, the rats were used for testing the response to carrageenin oedema by challenge with allergen containing one worm equivalent per μl . The larvae and allergen were gifts from Dr F.L. Pearce (Chemistry Department, University College, London).

The effectiveness of sensitization was determined from the ability of allergen to induce degranulation of peritoneal mast cells from sensitized rats (White & Pearce, 1982).

Materials

Peptide 401 was purified from freeze-dried bee venom (Bulgarcop, Sofia, Bulgaria) as described previously (Banks *et al.*, 1981). Compound 48/80 was from Sigma Chemical Co. (Poole, Dorset). Mepyramine maleate (Antihisan) was from May and Baker (Manchester). Methysergide maleate was from Sandoz (London) and cimetidine (Tagamet) was from Smith, Kline and French (Welwyn Garden City, Herts.). Carrageenin (sodium salt) was from BDH Chemicals Ltd. (Poole, Dorset).

Results

Inhibition of carrageenin oedema by peptide 401 and compound 48/80

Both peptide 401 and compound 48/80 suppress the normal response to subplantar carrageenin over a 4 h period, as shown in Figure 1a and c. Peptide 401 is more active, on a weight basis, showing significant activity at 0.1 mg kg^{-1} while compound 48/80 is active at 1 mg kg^{-1} . The dose-response curve for compound 48/80 is complicated because at higher doses (up to 15 mg kg^{-1}) the inhibition of swelling is less than it is at 2 mg kg^{-1} . There is also a considerable mortality among animals treated with compound 48/80 at doses higher than 2 mg kg^{-1} and we have, therefore, omitted doses of 10 and 15 mg kg^{-1} from Figures 1c and d.

The effect of a single sub-cutaneous dose of peptide 401 (1 mg kg^{-1}) on the response to carrageenin in animals depleted of histamine and 5-HT by pretreatment with compound 48/80 is shown in Figure 2. The reduction in swelling of the injected foot is much less in depleted animals than in normal animals but the swelling of the contralateral foot due to peptide 401 is greater, over the first 2 h, in the depleted animals than in normal animals. The same is true in depleted animals treated with single doses of compound 48/80 (15 mg kg^{-1}) as compared with non-depleted animals (data not shown).

Pretreatment with compound 48/80

Pretreatment of rats with compound 48/80 virtually eliminated the initial swelling in response to sub-plantar carrageenin but was without significant effect on the later stages (1.5 to 5 h). This is consistent with the observations made by Vinegar *et al.* (1976).

Effects of mepyramine and methysergide on the anti-inflammatory activities of peptide 401 and compound 48/80

Mepyramine (a histamine H_1 antagonist) and methysergide (a 5-HT antagonist) in doses that completely suppressed the skin responses to intradermal injection of histamine and 5-HT

Figure 1 The effects of peptide 401 (a, b) or compound 48/80 (c, d) at different doses on the swelling induced by sub-plantar carrageenin (0.1 ml, 1% w/v in 0.9% saline) in the right foot (a, c) and on the volume of the contralateral foot (b, d). Drugs were given s.c. in 0.1 ml 0.9% saline at the same time as the irritant ($t = 0$). Experimental points are the means of measurements in 5 animals; error bars denote $\pm$ s.e.mean and are included only for control and highest dose values for the sake of clarity. Standard errors did not exceed 20% of mean. (○) Control animals injected s.c. with 0.1 ml 0.9% saline; (●) 0.05 mg kg^{-1} ; (■) 0.1 mg kg^{-1} ; (▲) 1 mg kg^{-1} ; (▼) 2 mg kg^{-1} .

Figure 2 The effect of peptide 401 on the response to subplantar carrageenin in normal rats and in rats depleted of mast cell contents by pretreatment with compound 48/80 0.6 mg kg^{-1} twice daily for 3 days and 1.2 mg kg^{-1} twice on day 4. A single dose of peptide 401 (1 mg kg^{-1}) was given (s.c.) at the same time as carrageenin. Open symbols for volumes of uninjected (left) hind feet; filled symbols for injected feet. (○, ●) Control animals receiving 0.1 ml 0.9% saline, s.c.; (□, ■) normal animals receiving peptide 401; (△, ▲) depleted animals receiving peptide 401. Dashed lines, depleted animals, complete lines for normal animals. Experimental points are means of measurements from five animals and error bars are $\pm$ s.e.mean. Where not shown, standard errors did not exceed 20% of the mean.

Table 1 Inhibition of carrageenin-induced oedema in the rat hind paw by peptide 401 and by compound 48/80 and the effects of antagonists

Degranulator (mg kg ⁻¹)	Antagonist (mg kg ⁻¹)	n	t (h)	% Inhibition of oedema		
				1	2	4
—	H ₁ and 5-HT* (2.5)	5	13 ± 4.0		21 ± 4.0	9.5 ± 1.3
Peptide 401 (1)	—	15	-3 ± 0.27		50*** ± 5.0	50*** ± 9.2
401 (1)	H ₁ and 5-HT (2.5)	15	-27† ± 2.1		11†† ± 1.2	29†† ± 3.8
Compound 48/80 (10)	—	11	-30 ± 3.0		35*** ± 3.8	54*** ± 6.8
48/80 (10)	H ₁ and 5-HT (2.5)	10	-17 ± 2.5		11† ± 0.9	25†† ± 1.3
—	H ₂ ^b (50)	10	-14 ± 2.5		-4 ± 0.6	-5 ± 0.4
Peptide 401 (1)	—	10	37 ± 3.4		53 ± 4.4	53 ± 5.3
401 (1)	H ₂ (50)	10	63† ± 9.7		71† ± 14	67† ± 13
Compound 48/80 (10)	—	6	-28 ± 1.8		13 ± 0.8	36 ± 1.8
48/80 (10)	H ₂ (50)	5	3 ± 0.2		22 ± 1.7	40 ± 5.2

* Mepyramine and methysergide (H₁ and 5-HT antagonists respectively).

^b Cimetidine (H₂ antagonist).

Significance levels for drug compared with drug-free control (*) or antagonist compared with antagonist-free control (†). *** and †††, $P < 0.001$; ** and ††, $P < 0.005$; * and †, $P < 0.01$.

respectively (2.5 mg kg⁻¹ for each antagonist) reduced the anti-inflammatory activities of both peptide 401 and compound 48/80 by about 50% in animals when given 45 min prior to carrageenin injection. For the sake of clarity, only data obtained at 1, 2 and 4 h have been included in Table 1. The swelling of the contralateral paw was also suppressed (Table 2). Treatment with mepyramine and methysergide alone partly suppressed the initial phase of carrageenin oedema but did not significantly alter the later phase (Table 1). This observation is also consistent with the suggestions made earlier by Vinegar *et al.* (1976).

Effects of pretreatment with cimetidine on the anti-inflammatory activities of peptide 401 and compound 48/80

Pretreatment of rats with cimetidine (a histamine H₂-receptor antagonist) 45 min before carrageenin injection, slightly, though significantly ($P < 0.05$), enhanced the anti-inflammatory effects of peptide 401 and compound 48/80. Cimetidine alone (50 mg kg⁻¹) had no significant effect on the course of carrageenin oedema (Table 1).

Effects on carrageenin oedema of sensitization followed by allergen challenge

Rats that had been sensitized to the nematode, *Nippostrongylus brasiliensis*, were challenged subcutaneously with allergen, extracted from the worms, immediately following subplantar injection of carrageenin. The sensitized animals showed approximately 40% reduction in swelling compared

with control animals (Figure 3). In contrast to peptide 401 and compound 48/80, allergen challenge did not cause swelling of the contralateral paw. The anti-inflammatory activity resulting from allergen challenge was suppressed in sensitized

Figure 3 Effect of allergen challenge in rats sensitized to *N. brasiliensis* on the response to sub-plantar carrageenin. Complete lines are for normal animals; dashed lines for sensitized animals. Open symbols are for uninjected (left) hind feet; closed symbols for injected hind feet. (○, ●) Normal animals injected with saline (0.1 ml, 0.9%, s.c.) at the same time as carrageenin; (□, ■) normal animals injected with allergen (1000 worm equivalents, s.c.) at $t = 0$; (△, ▲) sensitized animals injected with saline; (▽, ▼) sensitized animals injected with allergen. Measurements are means on groups of five animals; error bars ± s.e.mean; where not shown, standard errors did not exceed 20% of the mean.

Table 2 Swelling in the uninjected paw induced by peptide 401 and compound 48/80 during the course of carrageenin-induced oedema and the effects of antagonists

Degranulator (mg kg ⁻¹)	Antagonist (mg kg ⁻¹)	n	t (h)	Increase in volume of uninjected foot (ml)				
			1	2	3	4	5	
Saline (control)	—	14	0.08 ± 0.02	0.09 ± 0.01	0.07 ± 0.02	0.09 ± 0.03	0.03 ± 0.03	
—	H ₁ and 5-HT* (2.5)	5	0.02 ± 0.04	0.01 ± 0.03	0.01 ± 0.04	0.01 ± 0.03	0.01 ± 0.02	
Peptide 401 (1)	—	15	0.26*** ± 0.02	0.27*** ± 0.03	0.19*** ± 0.03	0.21*** ± 0.04	0.17*** ± 0.05	
Peptide 401 (1)	H ₁ and 5-HT (2.5)	15	0.15† ± 0.02	0.15†† ± 0.02	0.13†† ± 0.02	0.13†† ± 0.03	0.11†† ± 0.03	
Saline (control)	—	10	0.03 ± 0.01	0.03 ± 0.01	0 ± 0.01	-0.02 ± 0.01	-0.03 ± 0.01	
Compound 48/80 (10)	—	11	0.23*** ± 0.02	0.21*** ± 0.02	0.16*** ± 0.02	0.15*** ± 0.02	0.16*** ± 0.02	
Compound 48/80	H ₁ and 5-HT (2.5)	10	0.11††† ± 0.02	0.08††† ± 0.03	0.03††† ± 0.02	0.02††† ± 0.02	0.02††† ± 0.02	

* Mepyramine and methysergide (H₁ and 5-HT antagonists respectively).

Levels of significance are for the effect of drug compared with drug-free control (*) and for the effect of inhibitor compared with inhibitor-free control (†), at significance levels of *** and †††, $P < 0.001$; ** and ††, $P < 0.005$; * and †, $P < 0.01$.

Figure 4 Increase in paw volume at 90 min following subplantar injections of peptide 401 (P401) (in 0.1 ml 0.9% saline) into the right hind feet of normal animals or animals pretreated 45 min previously with mepyramine (Mep) and methysergide (Methy) (2.5 mg kg^{-1}), or treated with peptide 401 (1 mg kg^{-1}) at the same time as sub-plantar injection of peptide 401. Measurements are means from groups of five animals; error bars $\pm$ s.e.mean.

animals pretreated with mepyramine and methysergide (each at 2.5 mg kg^{-1}) 45 min before carrageenin injection and allergen challenge (data not given). Animals tested before sensitization was complete (as judged by lack of degranulation of isolated mast cells induced by allergen *in vitro*) did not show a reduction in carrageenin-induced oedema when injected with allergen, as compared with saline-treated controls.

The irritant activity of peptide 401

Direct injection of peptide 401 into the rat hind paw produced swelling both in the injected (R) and uninjected (L) feet (Figure 4). Pretreatment of animals with mepyramine and methysergide as before almost abolished the swelling in response to subplantar 401 both in the injected and the uninjected paws. Sub-cutaneous injection of peptide 401 45 min before subplantar injection of the same peptide significantly suppressed the irritant action in the foot (Figure 4).

Co-administration of peptide 401 with carrageenin into the right hind paw produced a dose-dependent enhancement of the inflammatory response over the entire time course. The enhancement of swelling over the first 2 h is presumably due to the mast cell degranulating properties of the peptide, releasing vasoactive constituents locally. The effect of the peptide on the later phase of the oedema ($>2 \text{ h}$) is of more interest (see Discussion). The effect of co-injection of peptide 401 and carrageenin at 3 h is shown in Figure 5. The oedema produced by peptide 401 and carrageenin together was greater than the sum of the swellings produced by the irritants given separately (Figure 5).

Discussion

The results indicate that there is a strong connection between mast cell degranulation and suppression of carrageenin-induced oedema in the rat hind paw. This finding is in sharp contrast to earlier reports (Billingham *et al.*, 1973; Hanson *et al.*, 1974) in which the apparent anti-inflammatory effects of peptide 401 in a number of animal models were held not to depend on mast cell degranulation. The synthetic histamine releaser, compound 48/80, has now been shown to be nearly

Figure 5 Increases in paw volumes of uninjected (L) and injected (R) feet 3 h after injection into the hind paw of carrageenin (Carr) with and without addition of varying amounts of peptide 401 (P401). Volume injected, 0.1 ml, 1% carrageenin $\pm$ peptide 401 in 0.9% saline. Also shown is the increase in volumes of the hind feet 90 min after injection of the highest dose of peptide 401 alone. Measurements are means from groups of five animals; error bars $\pm$ s.e.mean.

as effective as peptide 401, in suppressing carrageenin-induced oedema but the dose-response curve is bell shaped, (see Results section). This could have led Hanson *et al.* (1974) to underestimate the anti-inflammatory activity of compound 48/80 in the earlier study. We have now shown that the activities of peptide 401 (and derivatives thereof) and of compound 48/80 (Figures 1a and c), parallel the abilities of these compounds to degranulate rat peritoneal mast cells *in vitro* (Banks *et al.*, 1980; Dempsey, 1981; present work). The activities of both peptide 401 and compound 48/80 were equally affected by such compounds as mepyramine, chlorpheniramine (results not shown), methysergide and cimetidine (Table 1).

Mast cell depletion results in a marked attenuation of the anti-inflammatory activity of both peptide 401 (Figure 2) and of compound 48/80 (results not shown). Pretreatment of rats with antagonists of histamine and of 5-HT also significantly reduced the anti-inflammatory effects of both degranulators (Table 1). Together, these findings suggest that the anti-inflammatory effects are at least in part mediated by mast cell-derived species. That depletion is rather more effective than is pretreatment with H_1 -receptor and 5-HT antagonists suggests that species other than histamine and 5-HT may be involved in the anti-inflammatory activity of mast cell degranulators. The slight enhancement of the anti-inflammatory effects of the two degranulators caused by pretreatment of animals with the H_2 -receptor antagonist, cimetidine (Table 1) is best understood as due to antagonism of feedback inhibition of mast cell degranulation by histamine acting on H_2 -receptors (Lichtenstein & Gillespie, 1975).

Vinegar *et al.* (1976) proposed that the early stage of carrageenin oedema in the rat hind paw was mediated by mast-cell

derived species. Consistently, we found in depleted animals that oedema formation in the first hour after injection of carrageenin was almost totally suppressed though the later phase (1.5–5 h) was unaffected (results not shown). On the other hand, neither H_1 nor 5-HT antagonists suppressed the early phase of oedema (Table 1) and this too is consistent with the work of Vinegar *et al.* (1982).

The effects of peptide 401 and of compound 48/80 on the uninjected feet require comment. At the same time as the swelling in response to sub-plantar carrageenin is reduced by these basic degranulators, injected sub-cutaneously, the contralateral feet swell. The effect is most marked in the early phase of the oedematous response in depleted animals (Figure 2). Were anti-inflammatory activity to be assessed from the difference in swelling of injected and uninjected feet (Billingham *et al.*, 1973; Hanson *et al.*, 1974) this might well give rise to an overestimate of activity. The origin of the swelling is obscure but might be associated with complexing of the basic degranulators with heparin that would also be released on degranulation in non-depleted animals and this could prevent access to the general circulation.

Atkinson & Hicks (1975) pointed out that true anti-inflammatory agents should always reduce the irritation produced by an inflammatory stimulus no matter how administered. 'Counter irritants', on the other hand, may reduce the inflammation at one site by producing an irritant effect elsewhere. To distinguish the two effects, it was noted that if a counter irritant were injected with, that is at the same site as a known irritant, inflammation would either be unaffected or enhanced whereas a true anti-inflammatory agent injected with the irritant should reduce the inflammatory response. It can be seen from Figure 5 that a subplantar

injection of peptide 401 together with carrageenin produces a considerable and dose-dependent enhancement of the oedematous response. The peptide is not, therefore, a true anti-inflammatory agent.

Final confirmation that mast cell degranulation can attenuate inflammation comes from experiments in rats sensitized to the nematode, *Nippostrongylus brasiliensis*. Subcutaneous allergen challenge at the same time as carrageenin is injected into the hind paw of sensitized animals reduces the swelling to the same extent as do the basic degranulators (Figure 3 compared with Figures 1 and 2). The only difference is that in the sensitized animals there is no swelling of the contralateral feet. This could be because the allergen remains bound, close to the site of injection and is not free to act systematically.

Mast cell degranulation may release chemotactic factors that mobilize or desensitize circulating neutrophils (Atkins *et al.*, 1978; Soter, 1980; Tannenbaum *et al.*, 1980) while access of the neutrophils to the site of inflammation (Atherton & Born, 1972) may be enhanced as a result of increased vascular permeability caused by histamine and 5-HT. Such a role for the biogenic amines would be consistent with the partial antagonism observed with mepyramine and methysergide.

It therefore appears that peptide 401 is a counter irritant. It reduces both the chemically mediated and cell-mediated phases of inflammation, being active in both the early and the later phases of carrageenin oedema and also against adjuvant arthritis (Billingham *et al.*, 1973) but it is most unlikely to be of any therapeutic value.

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