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MODIFICATION BY CAPSAICIN AND COMPOUND 48/80 OF DYE LEAKAGE INDUCED BY IRRITANTS IN THE RAT

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1 Concentration-related dye leakage produced by intracutaneous injections of irritants was measured in rats by an Evans blue technique.

2 In rats pretreated with a total dose of 50 mg capsaicin over 4 days, the response to capsaicin, formalin, HCl, KCl, prostaglandin E_1, bradykinin and bradykinin with prostaglandin E_1 (10^{-6} M) were greatly reduced, the responses to histamine and 5-hydroxytryptamine were slightly reduced and those to adenosine 5′-triphosphate (ATP) and compound 48/80 were unaffected.

3 Pretreatment with intracutaneous injections of compound 48/80 (0.5 μg, 24 and 48 h previously) recuded the responses to ATP, compound 48/80, HCl, KCl, prostaglandin E_1, and bradykinin but did not affect those to histamine, 5-hydroxytryptamine or bradykinin with prostaglandin E_1 (10^{-6} M).

4 Responses to capsaicin and formalin produced spotted blueing extending over a large area and were suppressed by compound 48/80 in the smaller pretreated area only. Capsaicin responses were reduced with larger doses of compound 48/80 (total dose 15 μg).

5 It is concluded that the production of neurogenic oedema involves both sensory nerves and mast cells.

Introduction

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), the pungent principle of chillies, has been shown to cause desensitization to chemically-induced pain, leaving sensitivity to mechanical stimuli unaffected (see Jancsó, 1960). Capsaicin pretreatment of animals also resulted in abolition of the inflammatory response to certain irritants and it was concluded that these irritants produced their inflammatory effects in untreated animals by stimulating 'pain receptors' to cause release of a neurohumour (Jancsó, Jancsó-Gábor & Szolcsányi, 1967, 1968). The object of the experiments on capsaicin-desensitized rats reported here was a more quantitative examination of the extent to which sensory nerve stimulation contributes to the oedema produced by a variety of irritants. Since Kiernan (1972a) has suggested that mast cells are involved in antidromic vasodilatation, another series of experiments was performed in which rats were pretreated with compound 48/80, to establish the role of mast cells as mediators of neurogenic oedema.

Methods

Oedema produced in rats by the intracutaneous injections of irritants was visualized by leakage of Evans blue from the circulation. Male Wistar rats, 100 to 150 g, were lightly anaesthetized with ether and the tail warmed in water at 45–50°C. Intravenous injections of a 2% solution of Evans blue in 0.9% w/v NaCl solution (saline), 2.5 ml/kg, were made into a lateral tail vein. Intracutaneous injections (0.05 ml) of the irritants tested were made on the abdominal skin shaved with commercial animal clippers fitted with a 1/10 mm shaving head. The conscious animals were left for 20 min at 25°C before killing by stretching the neck. After killing, the abdominal skin was cut out and pinned peritoneal side uppermost, to enable measurement of areas of blueing. Irritant substances tested were histamine, 5-hydroxytryptamine (5-HT), bradykinin, prostaglandins E_1, E_2 and $F_{2\alpha}$, acetylcholine, carbachol, HCl, KCl, adenosine 5′-triphosphate (ATP), capsaicin, compound 48/80, and a combination of bradykinin and prostaglandin E_1. Dilutions of irritants injected were made in Tyrode solution of the following composition (g/l): NaCl 8.0, KCl 0.2, $MgCl_2$ 0.1, $CaCl_2$ 0.2, NaH_2PO_4 0.05, $NaHCO_3$ 1.0. HCl and KCl were diluted in water to 0.15M (iso-osmotic), and further dilutions were made in Tyrode solution. For each substance tested, four doses of serial 1 in 2 dilutions and a Tyrode solution control were given, in 5 separate regions of the

abdominal skin. Five rats were used for each series with rotation of the doses to eliminate variation between sites.

To measure the intensity of the response, extraction of the dye was undertaken in a mixture of 7 ml of acetone and 3 ml of 0.5% sodium sulphate solution over 24 h according to the method of Harada, Takeuchi, Fukao & Katagiri (1971). After centrifugation the amount of dye was measured as absorbance at 620 nm using a Zeiss spectrophotometer. To allow for possible variations in background blueing of the rats, a reference blank for the readings was obtained from each rat by extraction as above of a similar sized piece of abdominal skin, taken from outside the blue areas.

Capsaicin pretreatment

Rats were pretreated with a 1% 'solution' of capsaicin made up in ethanol and Tween 80 according to the method described by Jancsó *et al.* (1967). This method produced a very fine suspension rather than a solution. Rats under ether anaesthesia were given 2.5 mg of capsaicin on the first day, and two doses of 5 mg in the morning and afternoon of the second day. Rats which experienced respiratory difficulty were given a dose of isoprenaline as an aerosol. Rats that survived the first doses (about 70% of animals) were given, without anaesthesia, a further 7.5 mg and 10 mg on the third day, and two doses of 10 mg on the fourth day, a total dose of 50 mg. Rats were tested with irritants on the fifth day. All injections of capsaicin were given subcutaneously on the back. Two control groups were pretreated with a solution that contained only Tween 80, ethanol and saline.

Compound 48/80 pretreatment

Rats under light ether anaesthesia were pretreated intracutaneously on the shaved abdominal skin with 0.05 ml of a 10 μg/ml solution of compound 48/80, 48 and 24 h before testing at the same sites with irritants (total dose of compound 48/80, 1 μg). One group was also pretreated with 100 μg/ml, 96, 72 and 48 h before testing with capsaicin (total dose of compound 48/80, 15 μg). One control group was pretreated intracutaneously with saline 48 and 24 h before testing with compound 48/80.

Analysis of results

Concentration-response lines were plotted by eye as absorbance against concentration on a logarithmic scale, through the mean responses at each concentration. During the course of the experiments, rapid assessment of the effect of treatments was obtained from *t* tests between the means of normal and pretreated groups at each concentration level. To obtain information on the overall effect of treatment on the concentration-response lines, two-way analysis of variance was used. Since it was apparent from the log concentration-response lines that some responses were fitted better by a curve than a straight line, partitioning of the concentration variance by the method of orthogonal contrasts was used to test the significance of curvilinear regression (the quadratic and cubic components). In those analyses where the interaction variance from the two-way analysis was significant, it was partitioned in a similar manner to determine whether there was any significant effect of treatment on the linear, quadratic or cubic components of the concentration variance.

Drugs

The following drugs were used: acetylcholine chloride (Sigma); adenosine 5′-triphosphate disodium salt (Sigma); bradykinin triacetate (Sigma); capsaicin (8-methyl-*N*-vanillyl-6-nonenamide) (Sigma); carbachol (BDH); compound 48/80 (Wellcome); Evans blue (Difco); histamine diphosphate (Sigma); hydrochloric acid, Analar, (Ajax); 5-hydroxytryptamine creatinine sulphate (Sigma); isoprenaline hydrochloride aerosol (Isuprel, Winthrop); potassium chloride, Analar, (Ajax) and prostaglandins E_1, E_2, $F_{2\alpha}$ tromethamine salt (Upjohn).

Results

Concentration-response lines for dye leakage could be obtained for all irritant substances tested except prostaglandins E_2 and $F_{2\alpha}$, acetylcholine and carbachol. Prostaglandin E_2 did not consistently produce significant dye leakage in the range 6×10^{-8} M to 6×10^{-5} M. Dye leakage could not be demonstrated at all with prostaglandin $F_{2\alpha}$ in the range of 6×10^{-7} M to 6×10^{-4} M. Therefore prostaglandins E_2 and $F_{2\alpha}$ were excluded from further study. Rats tested with acetylcholine 10^{-7} M to 10^{-1} M occasionally showed blueing at the lower concentrations but this was not a consistent finding. No obvious blueing was obtained with carbachol in the range 10^{-7} M to 10^{-3} M. Since the higher concentrations of acetylcholine and carbachol rapidly killed the rats, no further tests were made with these agents.

Control experiments

Tween 20, 40, 60 and 80 have been shown to be histamine liberators in the dog (Krantz, Carr, Bird & Cook, 1948), and 35% ethanol caused total histamine release from rat isolated mast cells (Bray & van Arsdel, 1961). Since the capsaicin suspension was made in Tween 80 and ethanol (see Methods section),

a control series of experiments was performed on rats pretreated with the same amount of saline, Tween 80 and ethanol as those that had undergone capsaicin pretreatment. No significant difference was obtained in the concentration-response lines to capsaicin and compound 48/80 in animals that had undergone such pretreatment. It was therefore assumed that any effects observed after capsaicin pretreatment were due to capsaicin and not to the suspending vehicle. Similarly, intracutaneous pretreatment with Tyrode solution in an identical manner to compound 48/80 pretreatment (see Methods section), did not significantly alter the response to intracutaneous testing with compound 48/80.

Visual appearance of responses

The visual appearance of the dye leakage response was qualitatively similar for most irritants except capsaicin and formalin. The dye leakage response to these substances extended over a larger area than that to the other irritants tested, though not across the midline of the abdomen, and was characterized by spotted blueing surrounding the area of injection.

Concentration-response lines

In both the capsaicin and compound 48/80 series of experiments the mean responses plotted against log concentration for most irritants were fitted adequately by a straight line, as shown by the lack of significant quadratic or cubic components of the concentration variances, and significant linear components: $P<0.001$ for all irritants except bradykinin ($0.1>P>0.001$ and $0.05>P>0.01$), HCl (non-significant regression in the capsaicin series and $P<0.001$ in the compound 48/80 series), KCl ($0.01>P>0.001$ and $P<0.001$) and prostaglandin E_1 ($0.05>P>0.01$ in the compound 48/80 series), each of which had shallow log concentration-response lines (Figures 1 to 4). The exception was compound 48/80 where the curvature of the line (Figure 4) was reflected in a significant quadratic component of the concentration variance ($P<0.001$ and $0.01>P>0.001$) as well as a significant linear component ($P<0.001$). Although the responses to ATP appeared to fit a curve (Figure 3), there was no significant quadratic component of the concentration variance in either series. However, it is likely that a quadratic component was

Table 1 Degree of reduction of dye leakage produced by pretreatment with capsaicin and compound 48/80

Irritant	*Concentration range (M)*	*Pretreatment*	*Degree of reduction§*	*Significance of components of interaction variance‡*	
				Linear	*Quadratic*
5-HT (Summer)	6.25×10^{-7} to	Capsaicin	†		
(Winter)	5×10^{-6}	48/80	0		
Histamine	5×10^{-5} to	Capsaicin	†		
	4×10^{-4}	48/80	0		
Bradykinin	1.25×10^{-6} to	Capsaicin	††		
	1×10^{-5}	48/80	††		
PGE_1 (1st Summer)	7.25×10^{-7} to	Capsaicin	†††	***	
(2nd Summer)	6×10^{-6}	48/80	†††	***	
Bradykinin with prostaglandin E_1 (10^{-6} M)	1.25×10^{-6} to 1×10^{-5}	Capsaicin 48/80	††† 0	**	
Compound 48/80 (0.25–2.0 μg/ml)	—	Capsaicin 48/80	0 ††	***	*
ATP	5×10^{-4} to	Capsaicin	0		
	4×10^{-3}	48/80	†††	***	*
HCl	7.5×10^{-4} to	Capsaicin	†††	**	
	6×10^{-3}	48/80	††		
KCl	1.25×10^{-2} to	Capsaicin	†††	**	
	1×10^{-1}	48/80	††		
Capsaicin	5×10^{-5} to	Capsaicin	†††	**	
	4×10^{-4}	48/80 (1 μg)	0		
		48/80 (15 μg)	††		
Formalin	3.62×10^{-2} to	Capsaicin	†††	**	
	3.0×10^{-1}	48/80	0		

§ Degree of reduction on arbitrary scale—0; none; †, slight; ††, marked; †††, very marked. (All reductions were significant at $P<0.001$.)

‡ The effect of pretreatments on the shape of the log concentration-response lines is shown in the significance of the linear and quadratic components of the partitioned interaction variance derived from two-way analysis of variance. *, $0.05>P>0.01$; **, $0.01>P>0.001$; ***, $P<0.001$.

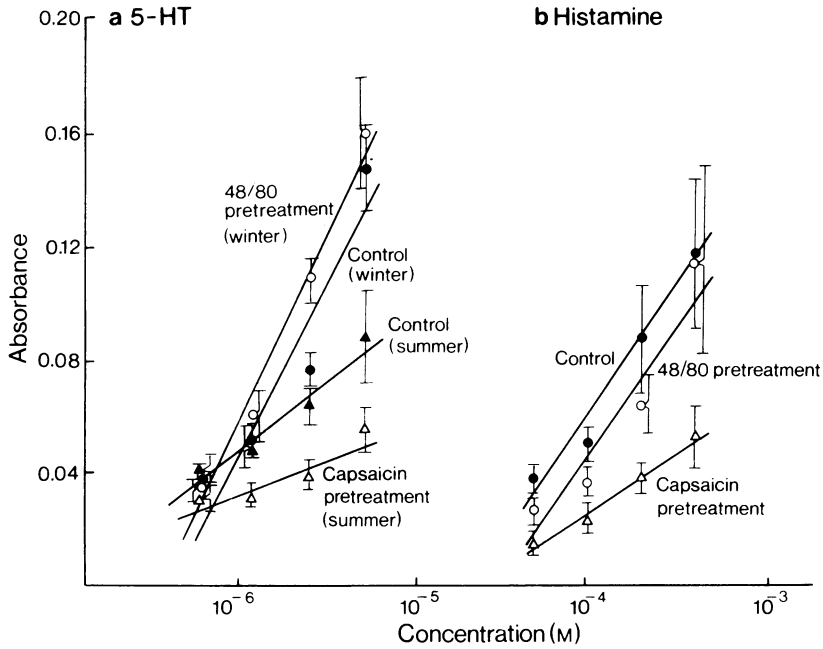

Figure 1 Rat skin responses to concentrations (M) of (a) 5-hydroxytryptamine (5-HT) and (b) histamine measured as Evans blue leakage (absorbance). Effect of pretreatment with capsaicin and compound 48/80. Means ± s.e. of 5 responses. Mean control (winter controls in (a)) responses (●); capsaicin pretreated (△); compound 48/80 pretreated (○). In (a), summer control responses (▲) were used for comparison with the capsaicin pretreatment responses. Lines plotted by eye. Concentration on a logarithmic scale.

present in the response to ATP since compound 48/80 pretreatment produced a significant effect on the quadratic component of the interaction variance ($0.05 > P > 0.01$) (Table 1).

The effects of pretreatment with capsaicin and compound 48/80 on the dye leakage response produced by the irritants are summarized in Table 1 and are shown graphically in Figures 1 to 4. Although a set of control concentration-response lines was obtained for both the capsaicin and the compound 48/80 series of experiments, and these were used in the respective statistical analyses, only one control line is shown in most figures, since there was no significant difference between the two control lines. An exception was 5-HT where there appeared to be marked seasonal variation in the control response, the concentration used producing more marked dye leakage in winter than in summer (Figure 1). Marked seasonal variation also occurred with prostaglandin E_1. A concentration-related response was obtained from 7.25×10^{-7} M to 6×10^{-6} M in summer, but no response could be shown from 10^{-8} M to 10^{-3} M in winter. The results shown in Figure 2 were obtained in two successive summers. A seasonal variation in the constrictor response to prostaglandin E_1 was found previously (Chahl & Ladd, 1976a). The responses to bradykinin were markedly potentiated by the addition of prostaglandin E_1 (1×10^{-6} M) to the solutions ($P < 0.001$). Two control lines are shown for bradykinin in the presence of prostaglandin E_1, since the two series of experiments were performed at different times of the summer season (Figure 2).

Capsaicin pretreatment

It can be seen that capsaicin pretreatment produced a partial reduction in the responses to 5-HT and histamine, a marked reduction in the responses to bradykinin and bradykinin with prostaglandin E_1, and almost complete abolition of the responses to prostaglandin E_1, HCl, KCl, capsaicin and formalin (Figures 1 to 4; Table 1). The abolition of the responses to the irritants was reflected in significant linear components of the interaction variances (see Table 1 for significance levels). The log concentration-response line to HCl from capsaicin-pretreated animals showed significant negative regression ($0.01 > P > 0.001$). These areas were blanched and this would explain the negative absorbance values compared with the blanks from skin outside the injected area. The responses to compound 48/80 and ATP were unaffected by capsaicin pretreatment (Table 1; Figures 3 and 4).

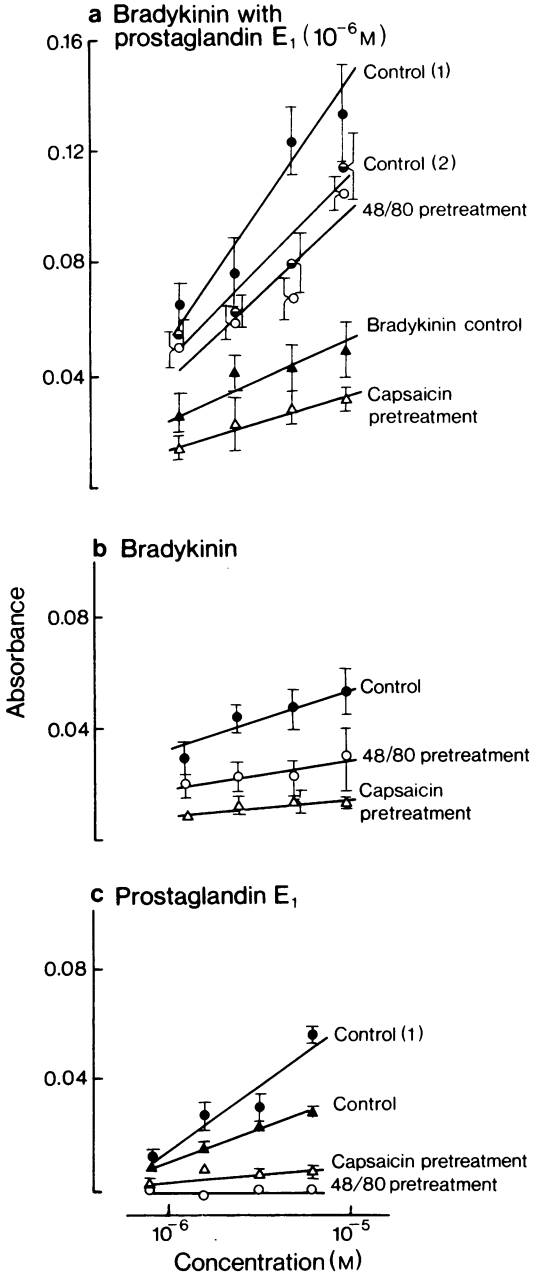

Figure 2 Rat skin responses to concentrations (M) of (a) bradykinin with prostaglandin E_1 (10^{-6} M) (b) bradykinin alone and (c) prostaglandin E_1 alone measured as Evans blue leakage (absorbance). Effect of pretreatment with capsaicin and compound 48/80. Means ± s.e. of 5 responses. Mean control (control (1) in (a) and (c)) responses (●); capsaicin pretreated (△); compound 48/80 pretreated (○). In (a), mean bradykinin responses (▲) were used for comparison with responses to bradykinin with prostaglandin E_1. Mean control (2) responses for bradykinin with prostaglandin E_1 (⊖) were used for comparison with compound 48/80 pretreatment responses (see text). In (c), mean responses to prostaglandin E_1 (▲) were used for comparison with compound 48/80 pretreatment responses (see text). Lines plotted by eye. Concentration on a logarithmic scale.

Compound 48/80 pretreatment

This pretreatment produced marked reduction in the responses to bradykinin, prostaglandin E_1, compound 48/80, ATP, HCl and KCl (Table 1, Figures 2, 3 and 4). Components of the interaction variance were significant for prostaglandin E_1, compound 48/80 and ATP (Table 1). It was observed that compound 48/80 pretreatment produced decreased blueing to capsaicin and formalin within the area of pretreatment, but outside this, the spotted blueing appeared more intense than that present in control animals. A group of rats pretreated with a higher dose of compound 48/80 (total dose, 15 μg) showed significant reduction in the total amount of dye leaked in response to capsaicin ($P < 0.001$) (Figure 4). The spotted blueing present in these pretreated animals extended over a similar area to that of the control animals but was much less intense.

Discussion

Capsaicin pretreatment which has been shown to block neurogenic inflammation (Jancsó *et al.*, 1967) appeared to provide a useful tool for examining the neurogenic component of oedema produced by those substances which cause cutaneous pain and which therefore must stimulate some sensory nerves. In our experiments rats subjected to the capsaicin pretreatment described by Jancsó *et al.* (1967) still showed scratching and lachrymation when drops of capsaicin were placed on the cornea, and dye leakage when injected intracutaneously. Therefore the amount of capsaicin used and the duration of pretreatment was increased. These pretreated rats showed no response to application of capsaicin to the eye and did not respond with dye leakage when capsaicin (5×10^{-5} M to 4×10^{-4} M) was given intracutaneously.

Both capsaicin and formalin when given intracutaneously produced a characteristic spotted blueing that did not extend across the midline of the abdomen. Other irritant substances tested produced a discrete circular area of blueing. The spotted blueing was similar in appearance to that observed by Chahl & Ladd (1976b) on antidromic stimulation of the saphenous nerve in rats. Therefore it would appear that spotted blueing is an indication of marked neurogenic oedema.

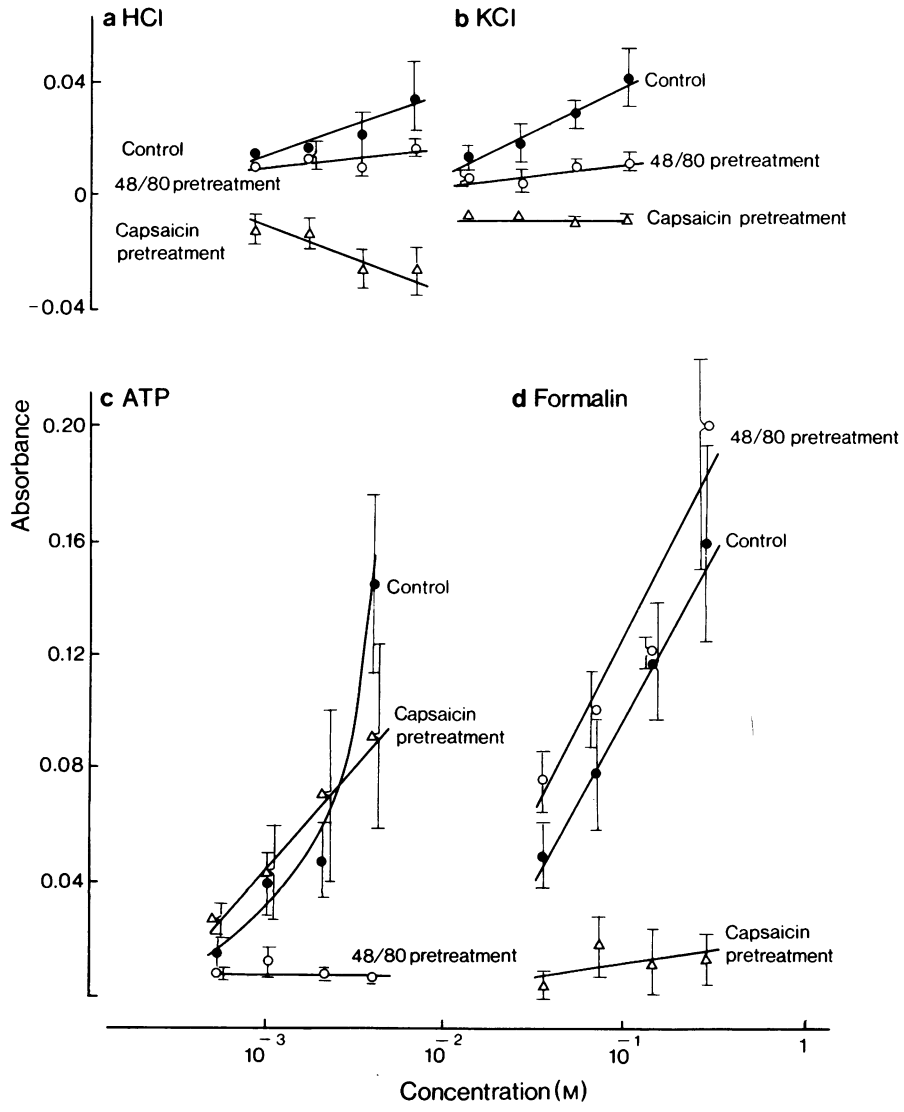

Figure 3 Rat skin responses to concentrations (M) of (a) HCl, (b) KCl, (c) adenosine 5'-triphosphate (ATP) and (d) formalin measured as Evans blue leakage (absorbance). Effect of pretreatment with capsaicin and compound 48/80. Means ± s.e. of 5 responses. Mean control responses (●); capsaicin pretreated (△); compound 48/80 (○). Lines plotted by eye. Concentration on a logarithmic scale. Negative absorbance values occurred with skin blanching (see text).

Since the dye leakage responses to capsaicin, formalin, HCl, KCl and prostaglandin E_1 were abolished after capsaicin pretreatment, it is postulated that these substances, in the concentrations tested, exerted their inflammatory effects via sensory nerve terminals. A neurogenic component of the inflammatory response has been previously demonstrated for capsaicin (Jancsó *et al.*, 1967) and formalin (Brown, Kissel & Lish, 1968). Histamine, 5-HT, bradykinin and bradykinin with prostaglandin E_1 still produced some dye leakage after capsaicin pretreatment, indicating that these agents produce oedema through some other mechanism in addition to a neurogenic mechanism. Compound 48/80 and ATP were unaffected by capsaicin pretreatment, showing that dye leakage due to these substances was independent of neurogenic mechanisms.

Jancsó *et al.* (1968) postulated that a 'neuro-

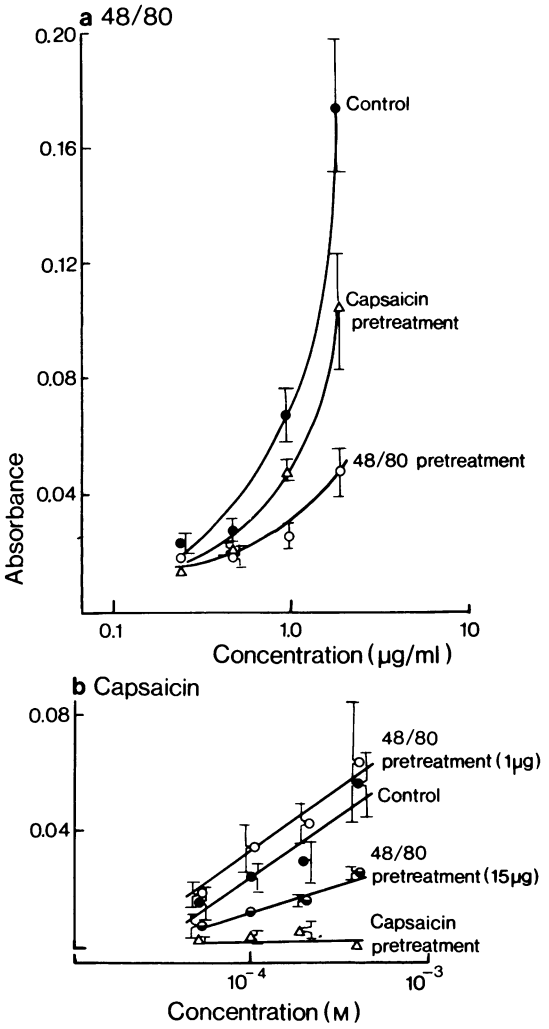

Figure 4 Rat skin responses to concentrations of (a) compound 48/80 (μg/ml) and (b) capsaicin (M) measured as Evans blue leakage (absorbance). Effect of pretreatment with capsaicin and compound 48/80. Means ± s.e. of 5 responses. Mean control responses (●); capsaicin pretreated (△); compound 48/80 pretreated (○). In (b), mean responses (◒) were obtained on pretreatment with a higher dose of compound 48/80 (15 μg). Lines plotted by eye. Concentration on a logarithmic scale.

humour' is liberated on stimulation of sensory nerves. Kiernan (1972a) has demonstrated the degranulation of mast cells following antidromic stimulation of cutaneous nerves, and therefore it is possible that this neurohumour might exert its effects by causing degranulation of mast cells. After pretreatment with a dose of compound 48/80 sufficient to decrease the dye leakage response to compound 48/80 itself, it was found that the responses to ATP and prostaglandin E_1 were abolished, confirming previous findings that these substances exert their effects by causing release of amines from mast cells (ATP—Diamant & Krüger, 1967; Kiernan, 1972b; prostaglandin E_1—Crunkhorn & Willis, 1971). The responses to bradykinin, HCl and KCl were also reduced suggesting that part of their action is mediated through mast cells, either directly or via the release of some neurohumour from sensory nerve terminals.

If some part of the action of a substance liberated from sensory nerves is via mast cells, it would be expected that, if dye leakage was greatly reduced by capsaicin pretreatment, then it would also be reduced by compound 48/80 pretreatment. This was found to be so for bradykinin, prostaglandin E_1, HCl and KCl. The exceptions to this were bradykinin with prostaglandin E_1, capsaicin and formalin. Capsaicin and formalin showed reduced blueing within the smaller area of compound 48/80 pretreatment, but enhanced spotted blueing outside this area. Pretreatment with a higher dose of compound 48/80 (15 μg) produced an overall reduction in intensity, but not in the area of response to capsaicin. It is possible that the enhanced blueing was due to the low dose of compound 48/80 being sufficient to lower the threshold of amine release from mast cells in the skin surrounding the pretreated area, but insufficient to produce depletion of amines. It is also possible that compound 48/80 or substances released by it might have a potentiating effect upon the action of amines released by the neurohumour from the incompletely depleted mast cells. This potentiating effect might also explain the lack of reduction in response to bradykinin with prostaglandin E_1 by compound 48/80 pretreatment. Nevertheless it is difficult to understand why responses to bradykinin and prostaglandin E_1 were each reduced by compound 48/80 pretreatment but the prostaglandin-potentiated bradykinin response was not. Prostaglandin E_1 has been shown to potentiate histamine as well as bradykinin responses (Moncada, Ferreira & Vane, 1973; Williams & Morley, 1973) and it is possible that the combination of bradykinin and prostaglandin E_1 produced so much more stimulation of the sensory nerves than either bradykinin or prostaglandin E_1 alone, and also enhancement of the effects of the amines released from the partially depleted mast cells, that the effect of compound 48/80 was masked. It is concluded that those irritants which produce neurogenic oedema cause release of one or more substances from sensory nerve terminals, and that at least part of the oedema produced is mediated by amines released from mast cells (Figure 5).

The mechanism of capsaicin densitization remains unclear. It is most probable that C fibres mediate neurogenic oedema (Chahl & Ladd, 1976b) and it would seem reasonable to suggest that any substance which produces pain should produce some component

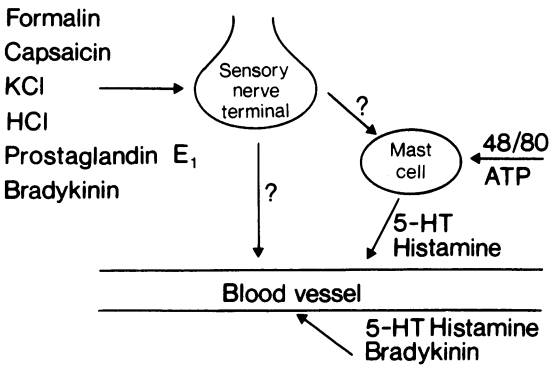

Figure 5 Suggested sites of action of irritants.

of neurogenic oedema. However it is not known whether the mechanism of release of the neurohumour from sensory nerve terminals is linked to the mechanisms involved in the production of action potentials in the nerve. In these experiments the lack of consistent dye leakage response to acetylcholine was unexpected since it has been shown to produce pain on the cantharidine blister base (Keele & Armstrong, 1964). It has been suggested that fluoride-resistant acid phosphatase in the Rolando substance of the spinal cord is functionally related to the processing of nociceptive stimuli (Knyihár, László & Tornyos, 1974) and since the activity of this enzyme disappeared completely after capsaicin pretreatment in the rat (Jancsó & Knyihár, 1975) it has been suggested that the effect of capsaicin on pain receptors is exerted at the level of the first neurone in the nociceptive pathway. The present findings could equally well be explained by an action of capsaicin at the level of the sensory nerve terminal.

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