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Substance P in sensory nerve fibres contributes to the development of oedema in the rat hind paw after thermal injury

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1 Immersion of the hind paws of anaesthetized rats in hot water for 5 min induced massive plasma protein leakage as indicated by extravasation of Evans blue dye in the skin. The threshold temperature which caused noticeable plasma extravasation was 45°C, a maximal response was obtained between 55°C and 60°C.

2 Pretreatment of rats 2 days after birth with 50 mg kg^{-1} capsaicin significantly reduced the Evans blue extravasation induced by hot water at 50°C, 55°C and 60°C, whereas guanethidine pretreatment 24 h before the experiment caused a significantly increased response at 40°C, 45°C and 50°C.

3 When Evans blue was injected between 10 and 120 min after immersion of the paw in hot water, a significant extravasation of the dye was no longer detectable. However, the weight of the paw as well as the weight of the piece of skin taken for Evans blue quantification increased during this period indicating the progressive development of oedema in the skin and underlying tissues.

4 In rats treated with capsaicin as neonates, the increase in paw weight after immersion in water of 50°C for 5 min was significantly delayed during the first hour, but there was no difference after two hours.

5 In rats pretreated with D-Arg^1,D-Pro^2,D-$Trp^{7,9}$, Leu^{11}-substance P, a substance P (SP) antagonist, the Evans blue extravasation was significantly reduced. However, the response, which remained in rats treated with capsaicin as neonates was not blocked by the SP-antagonist.

6 It is concluded that activation of peripheral branches of sensory SP neurones contributes to the initial massive protein extravasation and to the subsequent rate of development of oedema following heat injury. Release of histamine did not significantly contribute to this response at the lower temperatures, although the response was reduced by histamine receptor blocking drugs at 55°C and 60°C. Decreasing the sympathetic vasoconstrictor tone by guanethidine resulted in an increased response.

Introduction

Oedema formation induced by thermal injury has been frequently investigated (see Davies, 1982). The release or local formation of several mediators involved in this process have been described, i.e. 5-hydroxytryptamine (5-HT), histamine (Rosenthal *et al.*, 1957, Spector & Willoughby, 1958), kinins including bradykinin (Starr & West, 1967) and prostaglandins E_1, E_2 and $F_{2\alpha}$ (Heggers *et al.*, 1980).

Vascular protein leakage after stimulation of peripheral nerves is caused by release of a mediator from the peripheral terminals of C-fibre afferents, since it was shown to be abolished after pretreatment of newborn rats with capsaicin, which leads to a selective degeneration of certain populations of sensory C-fibres (Jancsó *et al.*, 1977). The neurogenic mediator causing vascular protein leakage was identified as substance P (SP) in the skin (Lembeck & Holzer, 1979, Lembeck *et al.*, 1982) and in the respiratory tract (Lundberg & Saria, 1983, Lundberg *et al.*, 1983). The aim of the present study was to investigate the importance of sensory SP neurones in the development of oedema after thermal injury at different temperatures using capsaicin pretreatment and a SP antagonist.

Methods

Animals

Sprague-Dawley rats of either sex (strain OFA-SD, Himberg, Austria) weighing between 200 and 300 g were used for all studies.

Thermal injury

The animals were anaesthetized with pentobarbitone sodium (40 mg kg^{-1}, i.p.). Evans blue (30 mg kg^{-1}) was injected into a jugular vein. After 5 min, the right hind paw was immersed in hot water to a level just above the tibiotarsal joint for 5 min. The water temperature was controlled by a thermostat. For time course studies, the paws were immersed for 5 min in hot water and Evans blue was injected into a jugular vein either immediately before or at different times following the immersion (see Figures 2 and 3). At the end of the experiment the rats were exsanguinated and the hind paws were cut off at the tibiotarsal joint.

Quantification of oedema and plasma protein leakage

To measure the magnitude of the oedema the paws were weighed and the ratio of paw weight: body weight was calculated (see Davies, 1982). To assess vascular protein leakage, the dorsal paw skin was removed, extracted with formamide and the content of Evans blue was estimated as described by Saria & Lundberg (1983a).

Drug pretreatments

Capsaicin was administered to neonatal rats (2nd day of life) in a dose of 50 mg kg^{-1}, subcutaneously, under ether anaesthesia as described by Donnerer & Lembeck (1983) to destroy capsaicin sensitive afferent nerve fibres (Jancsó *et al.*, 1977). For blocking histamine H_1- and H_2-receptors, diphenhydramine (10 mg kg^{-1}) and ranitidine (2 mg kg^{-1}) were given intraperitoneally 30 min before heat exposure. This dose reduced the Evans blue extravasation induced by histamine (1 mg kg^{-1} i.v.) to less than 10% in the

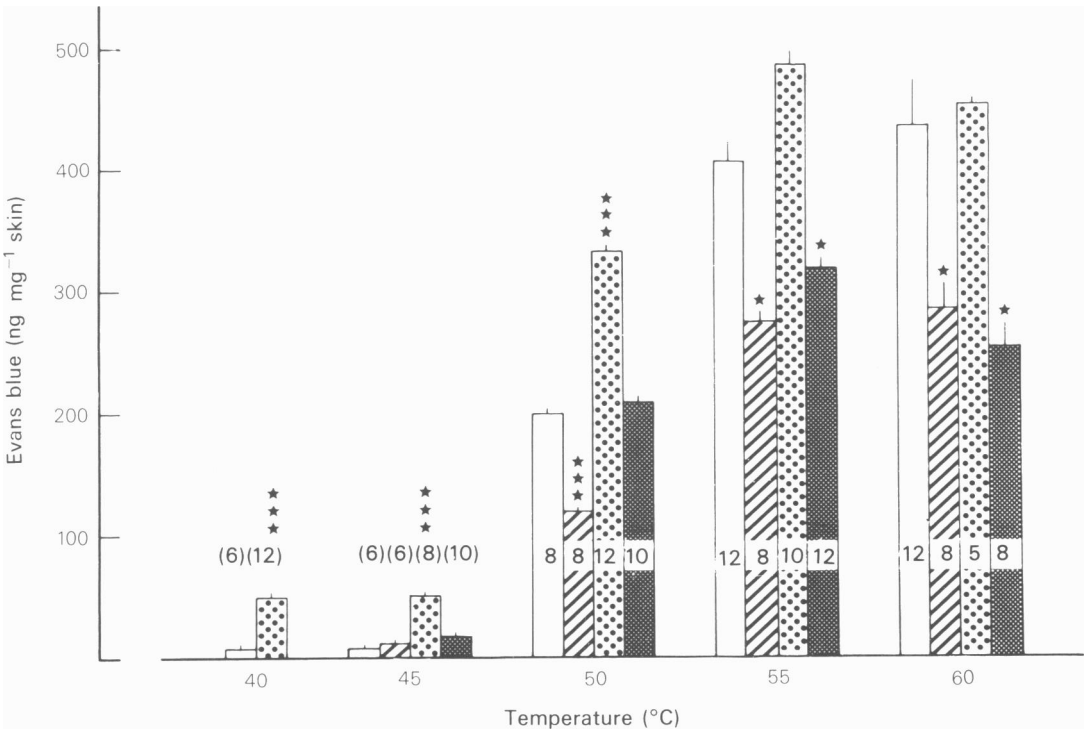

Figure 1 Evans blue extravasation into the skin of the hind paw of anaesthetized rats during immersion in hot water for 5 min at the different temperatures indicated. Open columns: control rats, hatched columns: rats treated with capsaicin as neonates (50 mg kg^{-1}, s.c.); large dots: rats pretreated with guanethidine (20 mg kg^{-1} i.p.) 24 h before the experiment; small dots: rats pretreated with diphenhydramine (10 mg kg^{-1}, i.p.) plus ranitidine (2 mg kg^{-1}, i.p.) 30 min before the experiment. Results are expressed as ng Evans blue per mg skin, mean values ± s.e.mean (vertical bars); numbers above or in the columns = number of animals. Significantly different from untreated controls: $^{*}P < 0.05$ and $^{***}P < 0.001$ (one way analysis of variance and Duncan's multiple range test).

paw skin and in several visceral organs of the rat (data not shown). This effect of diphenhydramine and ranitidine resembles the histamine blocking effect of a similar dose of mepyramine and cimetidine (Saria *et al.*, 1983). The SP antagonist D-Arg[1],D-Pro[2],D-Trp[7,9],Leu[11]-substance P was infused intravenously at a rate of 0.1 mg kg^{-1} min^{-1} for 10 min (total volume 1.0 ml). The specificity of this SP antagonist was described by Rosell *et al.*, 1983 and, with respect to neurogenic inflammation, by Lundberg *et al.*, 1983. An i.p. injection of guanethidine (20 mg kg^{-1}) was given 24 h before heat exposure. Doses refer to the free base.

Drugs

Capsaicin (Merck, Darmstadt, FRG), D-Arg[1],D-Pro[2],D-Trp[7,9],Leu[11]-substance P (Bachem, Bubendorf, Switzerland), guanethidine sulphate (Ciba-Geigy, Basle, Switzerland), ranitidine hydrochloride (Gebro, Fieberbrunn, Austria) and diphenhydramine hydrochloride (Chemofux, Vienna, Austria) were used.

Statistics

For statistical analysis one way analysis of variance and Duncan's multiple range test were used when different treatments were compared with the same control group; when individual groups were compared, Student's two sample *t* test was used.

Results

Effect of drugs on protein leakage into the skin of the rat paw during immersion in heated water for 5 min

The skin of hind paws of anaesthetized control rats showed a temperature dependent extravasation of Evans blue during immersion in hot water (Figure 1). The threshold temperature, which caused a noticeable diffusion of Evans blue into the paw skin was 45°C. The response increased at higher temperatures, reaching a plateau between 55°C and 60°C. In capsaicin pretreated animals, Evans blue extravasation was significantly reduced at 50°C, 55°C and 60°C, by 40%, 31% and 34%, respectively (Figure 1). Guanethidine pretreatment enhanced the response at 45° and 50°C by 220% and 70%, respectively; the responses at 55°C and 60°C were unchanged. In guanethidine pretreated animals, a significant extravasation of Evans blue was observed at 40°C (Figure 1). Blocking both types of histamine receptors with diphenydramine and ranitidine resulted in a reduction in the extravasation of Evans blue at 55°C and 60°C (21% and 43% decrease) but not at lower temperatures (Figure 1). Evans blue extravasation was also visible in deeper structures, but was not quantified there.

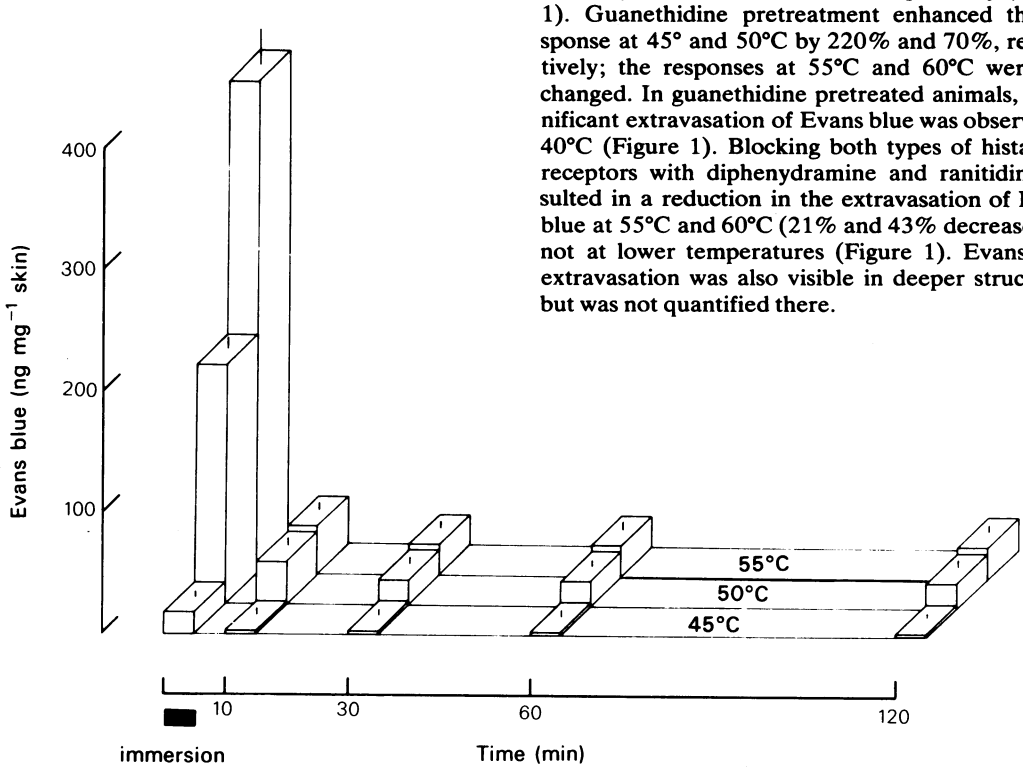

Figure 2 Extravasation of Evans blue during immersion of the hind paw of the rat in hot water of different temperatures for 5 min, or during a 5 min period following injection of Evans blue 10, 30, 60 or 120 min after onset of the immersion. Ordinate scale: Evans blue content of the skin in ng per mg skin (mean values ± s.e.mean (vertical bars); $n = 6$). Abscissa scale: time of injection of Evans blue after onset of immersion.

Protein extravasation and oedema formation during a period of two hours following a 5 min immersion in hot water

Evans blue was injected immediately before and at different time intervals following a 5 min immersion of the paw of control rats in hot water; the rats were killed 5 min after the injection. From Figure 2 it can be seen that the largest protein leakage occurred during the immersion. When Evans blue was injected 10, 30, 60 or 120 min later no large protein extravasation occurred, even at 60°C. In contrast to the protein extravasation, the paw weight increased with time, reaching a maximum after about 2 h at 50°C and after 30 min at 55°C (Figure 3). In capsaicin treated rats the increase in paw weight was delayed when the water temperature was 50°C (Figure 3). However, after 2 h the paw weights in capsaicin pretreated animals were similar to those of normal animals.

Effect of D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}-substance P on initial Evans blue extravasation

Intravenous infusion (1 mg kg^{-1}) of the SP antagonist D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}-SP 10 min immediately before paw immersion at 50°C significantly reduced (−31%) the extravasation of Evans blue during the 5 min of immersion (Figure 4). The reduced Evans blue extravasation occurring in capsaicin pretreated animals was not affected by infusion of 1 mg kg^{-1} of D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}-SP.

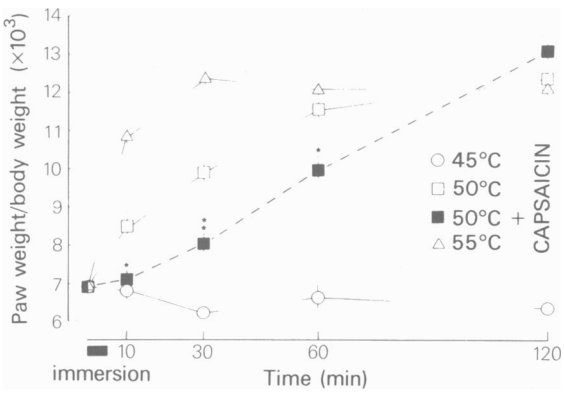

Figure 3 Formation of oedema in the hind paw of the rat induced by immersion in hot water ((○) 45°C, (□) 50°C, (△) 55°C) for a period of 5 min in normal rats and in rats treated with capsaicin (50 mg kg^{-1} as neonates ((■) 50°C + capsaicin). Ordinate scale: paw weight: body weight (mean values ± s.e.mean (vertical bars), $n = 6$ for each group). Abscissa scale: time after start of immersion in hot water. Significant difference between normal and capsaicin treated rats at a water temperature of 50°C: * $P < 0.05$, ** $P < 0.01$ (Student's two sample t test).

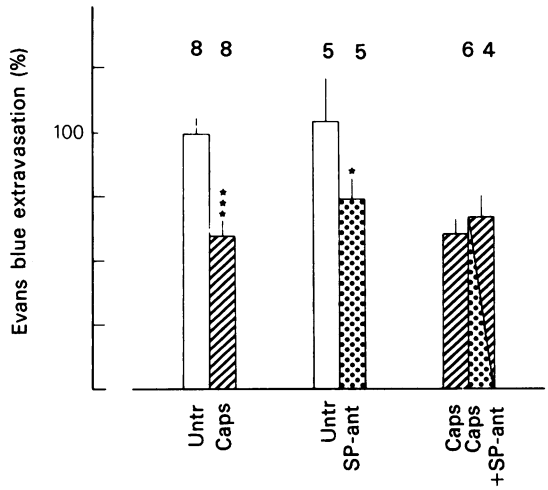

Figure 4 Extravasation of Evans blue into the skin of the hind paw of the rat during a 5 min immersion in water at 50°C. Responses are expressed as % of the response in the untreated control group which was compared with the capsaicin-treated group. Untreated rats (Untr); rats pretreated with the substance P (SP) antagonist (i.v. infusion for 10 min at 0.1 mg kg^{-1} min^{-1}) (SP-ant); rats pretreated with capsaicin (50 mg kg^{-1} as neonates) (Caps) and rats pretreated with capsaicin and the SP antagonist (Caps + SP-ant). Significantly different from untreated controls (individual groups): * $P < 0.05$, *** $P < 0.001$ (Student's two sample t test). Results are expressed as mean (%) ± s.e.mean (vertical bars) of a number (above columns) of animals.

The dose of the SP antagonist used was the highest one which did not by itself produce extravasation of Evans blue.

Discussion

It is well established that the inhibitory effect of capsaicin treatment of neonatal rats on plasma extravasation following chemical irritation is due to degeneration of sensory C-fibres. The present data indicate that sensory neurones which are sensitive to capsaicin may contribute to the large initial protein extravasation caused by heat. They confirm previous observations made using a water temperature of 48°C (Saria & Lundberg, 1983b). The degree of inhibition by capsaicin pretreatment was similar at all temperatures investigated which indicates that the contribution of sensory neurones was independent of the severity of injury in the observed temperature range. The peak of the protein extravasation which occurred during immersion for 5 min, was followed by an increase in the weight of the paw due to oedema formation. This proceeded without further extrav-

asation of plasma proteins in the skin. The increase in the weight of the paws was accompanied by an increase in the weight of the skin area taken for Evans blue quantification. However, this increase (from approximately 100 mg to approximately 150–200 mg after 2 h, data not shown) cannot explain the doubling of the weight of the paws during this period. Thus, it is assumed that fluid accumulation occurred in the skin as well as in underlying structures. In capsaicin treated animals, the fluid accumulation was delayed, but the oedema reached the same magnitude as in untreated animals 2 h after the injury was produced. However, it cannot be established whether this delay was caused by the reduced initial protein extravasation (see Figure 1) and thus by altered osmotic conditions, or whether sensory mechanisms can influence fluid accumulation in the later phase. There is good evidence that SP is the transmitter in the sensory nerves involved in the neurogenic plasma extravasation (Lembeck & Holzer 1979, Lembeck *et al.*, 1982, Lundberg *et al.*, 1983). This is supported by the observation that the substance P antagonist, D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}-SP inhibited the Evans blue extravasation induced by heat. The observation that the non-neurogenic plasma extravasation which remained in capsaicin treated rats (see Figure 4) could not be antagonized by this substance is good evidence that it is a specific antagonist for SP. Histamine release by substance P (Lembeck & Holzer, 1979) does not contribute significantly to the heat-induced plasma extravasation since histamine receptor blockade did not reduce protein extravasation at 50°C, a temperature at which capsaicin pretreatment did inhibit the response (see Figure 1). The release of histamine at higher temperatures (Rosenthal *et al.*, 1957) was confirmed by the observation that plasma extravasation at 55°C and 60°C could be antagonized by histamine receptor blockade.

Protein extravasation during thermal injury was enhanced by pretreatment with guanethidine indicating that sympathetic vasoconstrictor fibres are involved in this response (Cooper *et al.*, 1963). The assumption that the control of vascular permeability is by sympathetic fibres is supported by the inhibitory effect of noradrenaline (Marciniak *et al.*, 1978), although we do not know the exact mechanism of the inhibitory action of catecholamines on the increase in vascular permeability induced by inflammatory agents. At 55°C and 60°C plasma extravasation was probably maximal, which would explain the lack of effect of guanethidine.

SP release causing plasma protein leakage could be due to a direct activation of sensory nerve endings by heat (Fleischer *et al.*, 1983). In this context it is interesting to note that the threshold temperature for heat-induced plasma extravasation was 45°C which is identical with the threshold for excitation of polymodal nociceptors (see Fleischer *et al.*, 1983). This further indicates the involvement of sensory neurones and supports the hypothesis of Celander & Folkow (1953) that only nociceptive afferent fibres are provided with the axon reflex arrangement responsible for neurogenic inflammation. Heat could, dependent on the severity of injury, also cause the local release of substances such as histamine, bradykinin or 5-HT which subsequently stimulate SP fibres (Jancsó *et al.*, 1980). Since peripheral branches of sensory SP neurones may be involved in neurogenic inflammation of the skin in man (Anand *et al.*, 1983), it will be of interest to determine whether inhibition of SP receptors or blockade of SP release could be useful as initial treatment of oedema caused by thermal injury in man.

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