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Involvement of sensory neuropeptides in the development of plasma extravasation in rat dorsal skin following thermal injury

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1 The involvement of the neuropeptides, substance P (SP) and calcitonin gene-related peptide (CGRP) in plasma extravasation following thermal injury of rat dorsal skin was investigated.

2 Heat applied to the dorsal skin of anaesthetized rats by a temperature-controlled skin heater (1 cm diameter) for 5 min induced temperature-dependent plasma protein extravasation at 46°C to 50°C measured over the 20 min following initiation of heat.

3 The NK_1-receptor antagonist, SR140333, at doses above 36 nmol kg^{-1}, significantly ($P<0.05$) inhibited plasma extravasation by up to $79\pm3\%$ (120 nmol kg^{-1}) after heat application at 48°C and by up to $53\pm10\%$ (120 nmol kg^{-1}) after heat application at 50°C.

4 The $CGRP_1$-receptor antagonist, $CGRP_{8-37}$, at doses of 200 and 400 nmol kg^{-1}, significantly inhibited ($P<0.01$) plasma extravasation by 55 ± 9 and $60\pm12\%$, respectively, after heat application at 48°C. At a dose of 200 nmol kg^{-1} $CGRP_{8-37}$ inhibited plasma extravasation by $41\pm8\%$ after heat application at 50°C.

5 SR140333, 120 nmol kg^{-1}, and $CGRP_{8-37}$, 200 nmol kg^{-1} together significantly ($P<0.01$) inhibited plasma extravasation by $84\pm15\%$ after heating at 48°C for 5 min.

6 In experiments where the response was measured for 0-5, 5-10, 10-15 or 15-20 min, SR140333, 120 nmol kg^{-1}, significantly ($P<0.05$) inhibited plasma extravasation which had accumulated during all the time periods measured. In comparison, $CGRP_{8-37}$, 200 nmol kg^{-1}, was significantly ($P<0.05$) effective at time-points up to 15 min after initiation of injury.

7 In longer term experiments plasma protein extravasation continued for at least 95 min after initiation of thermal injury. SR140333, at a dose of 120 nmol kg^{-1}, significantly inhibited plasma extravasation for up to 65 min after initiation of injury.

8 In conclusion, the data from the present study demonstrate that both SP and CGRP are likely to have a role in the acute plasma extravasation after thermal injury. In addition, evidence suggests SP may have a role in plasma extravasation for up to 65 min.

Keywords: Thermal injury; plasma extravasation; dorsal rat skin; CGRP; substance P

Introduction

It is well established that oedema formation is induced by thermal injury, the magnitude of which depends on the temperature and duration of the burn (Saria, 1984; Blomgren & Bagge, 1984). Several mediators have been shown to be involved in the initial inflammatory process, i.e., histamine (Rosenthal *et al.*, 1957), bradykinin, (Rocha e Silva & Rosenthal, 1961; Starr & West, 1967), 5-hydroxytryptamine, prostaglandins (Willis, 1970; Jonsson, 1971; Jonsson *et al.*, 1979; Williams, 1979) and substance P (SP; Saria, 1984; Jonsson *et al.*, 1986). Furthermore, Saria (1984) showed that pretreating neonatal rats with capsaicin to cause selective degeneration of sensory C-fibres significantly inhibited plasma extravasation in the rat hind paw following thermal injury.

Antidromic electrical stimulation of sensory nerves, as well as chemical irritants or noxious heat, leads to plasma protein extravasation and vasodilatation (Jancso *et al.*, 1977) mediated by the release of neuropeptides such as SP and calcitonin gene-related peptide (CGRP). It has been proposed that oedema formation is dependent upon both the presence of a vasodilator and an agent which increases vascular protein leakage. Moreover, it has been suggested that the presence of these two factors together will act synergistically to produce a greater oedema response than that produced when either is present alone (Brain & Williams, 1985). SP and CGRP have been shown to be co-localized in sensory neurones (Gibbons *et al.*, 1985; Lundberg *et al.*, 1985). SP is a potent mast cell activator and increases microvascular permeability directly by acting on NK_1-receptors (see Regoli *et al.*, 1994). CGRP is a potent vasodilator which acts via $CGRP_1$-receptors to cause prolonged vasodilatation (Brain & Williams, 1985; Escott & Brain, 1993). In rat skin, evidence suggests that after sensory nerve stimulation the resulting oedema can be totally inhibited by NK_1 receptor antagonists (Lembeck & Holzer, 1979; Lembeck *et al.*, 1982) and partially inhibited by a $CGRP_1$-receptor antagonist (Escott & Brain, 1993). This together with other supporting studies suggest that NK_1-dependent receptor mechanisms are primarily responsible for the increased vascular permeability and CGRP for the vasodilatation. More recently, it has been shown that during thermal stimulation of the rat hind paw, there is a marked increase in both the release of immunoreactive SP (Yonehara *et al.*, 1987) and CGRP (Yonehara *et al.*, 1991), but not of neurokinin A (Yonehara *et al.*, 1991).

Therefore, in this study we have investigated the role of both the sensory neuropeptides, SP and CGRP, in the initial response to local cutaneous burn injury using antagonists which have been shown to be both selective and effective in rat skin; SR140333, an NK_1-receptor antagonist (Emonds-Alt *et al.*, 1993) and the $CGRP_1$-receptor antagonist, $CGRP_{8-37}$ (Donoso *et al.*, 1990; Gardiner *et al.*, 1990; Escott & Brain, 1993). In addition, we have studied whether the plasma extravasation is an ongoing process and if SP has a role in this later phase.

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Methods

Animals

Male Wistar rats (250–280 g) were anaesthetized with sodium pentobarbitone (Sagatal; 50 mg kg^{-1}, i.p.) with maintenance doses administered as required (i.v.). Their dorsal skin was shaved and depilated with a commercial cream (Immac, Reckitt & Coleman, Hull; 5 min) and all rats were left for at least 30 min after removal of the depilatory cream before heat was applied. The tail vein was cannulated for the administration of agents under study and sodium pentobarbitone.

Response to local thermal injury

Local cutaneous thermal injury was induced with a Moor (Devon, UK) temperature-controlled skin heater (1 cm diameter) along the midline area of the dorsal skin. ^{125}I-labelled human serum albumin (HSA; 2.5 μCi per rat) and Evan's blue dye (as a visual aid; 300 μl 2.5% in saline) were injected i.v. 5 min prior to heating. In initial experiments heat was applied to the skin for 5 min at a range of temperatures between 44°C to 50°C and plasma accumulation was allowed for 20 min after initiation of thermal injury. In some animals the NK_1 antagonist, SR140333 and/or the CGRP antagonist, $CGRP_{8-37}$ was administered i.v., 5 min prior to application of the skin heater.

In experiments designed to study the time-course of the response, heat (48°C) was applied to the dorsal skin for 5 min. [^{125}I]-HSA and Evan's blue were then injected at different time-points following the injury (5, 10 or 15 min) and accumulation measured for the following 5 min (i.e. 5–10, 10–15, 15–20 min). Antagonist or vehicle was added 5 min prior to [^{125}I]-HSA and Evan's blue.

In a separate series of experiments to study later time-points of the response, heat (48°C) was applied to the dorsal skin for 5 min and [^{125}I]-HSA and Evan's blue were then injected at different time-points following the injury (5, 35 or 65 min) and measured for the following 30 min period (i.e. 5–35, 35–65, 65–95 min). SR140333 was injected either 5 min prior to application of the skin heater (−5 min) or in some experiments 5 min prior to [^{125}I]-HSA and Evan's blue at different time-points.

Response to local intradermally injected agents

In a separate series of experiments designed to test the selectivity of SR140333 for NK_1 receptors, agents were made up in Tyrode solution and 100 μl volumes were injected intradermally into rat dorsal skin. The response was measured as the extravasation of intravenously injected [^{125}I]-HSA accumulated at skin sites over 30 min as previously described (Brain & Williams, 1985).

Measurement of plasma protein extravasation

At the end of the accumulation period, a cardiac blood sample (1.5 ml) was taken and the animal killed by anaesthetic overdose followed by exsanguination. The blood sample was centrifuged at 8000 *g* for 4 min to obtain a plasma sample. The dorsal skin of the rat was removed and the inner surface cleaned of any blood. The thermal injury sites and two control unheated sites, or the intradermal injection sites, were punched out and their radioactivity counted along with a 100 μl sample of plasma. Plasma extravasation was expressed as the volume of plasma accumulated in skin sites compared to total radioactivity present in 1 ml of plasma.

Drug treatments

SR140333 and $CGRP_{8-37}$ in 0.9% saline were given i.v. in 1 ml kg^{-1}. For blocking NK_1 receptors, SR140333 (12–360 nmol kg^{-1}) was administered either 5 min before thermal injury or for some of the time course experiments 5 min prior to administration of [^{125}I]-HSA and Evan's blue at various time points after the thermal injury. To block $CGRP_1$ receptors, $CGRP_{8-37}$ (200, 400 nmol kg^{-1}) was administered 5 min prior to application of the skin heater or 5 min prior to administration of [^{125}I]-HSA and Evan's blue at various time points after the cessation of heat. Vehicle controls for the two antagonists were administered to different groups of animals, 0.1% ethanol and 0.01% bovine serum albumin for SR140333 and $CGRP_{8-37}$ respectively.

Statistical analysis

Results are expressed as mean ± s.e. mean. Statistical analysis was carried out on the raw data which had been logarithmically transformed to give a normal distribution. This was because the s.e. means were positively correlated to the mean and the sample size was too small for non-parametric statistical tests. Differences between thermal injury sites at one time-point in vehicle-treated and drug-treated animals were compared by Student's *t* test for unpaired data. For experiments comparing unheated with heated sites on the same rat, differences were assessed by one-way analysis of variance followed by Dunnett's test. All other data were assessed by one-way analysis of variance followed by Bonferroni's test. Significance was taken as $P<0.05$.

Materials

^{125}I-labelled human serum albumin was obtained from Amersham, Amersham UK and Sagatal from Rhone Merieux, Dublin. Substance P, bradykinin and histamine were purchased from Sigma, UK, $CGRP_{8-37}$ from Peninsula, St. Helens, UK and CGRP from Bachem. SR140333 ((S)1-{2-[3(3,4-dichlorophenyl)-1-(3-iso-propoxyphenylacetyl)piperidine-3-yl]ethyl}-4-phenyl-1-azoniabicyclo[2.2.2]octane chloride) was a gift from Sanofi, Toulouse, France and GR73632 (δAva[L-Pro^9,N-Me-Leu^{10}]SP(7–11)) was a gift from Glaxo Wellcome, UK.

Results

Local heating of rat skin for 5 min gave a temperature-dependent response with respect to plasma extravasation when measured over 0–20 min. Heating skin at 44°C had no significant effect on plasma extravasation in dorsal skin. However, heating at 46°C, 48°C and 50°C gave a significant ($P<0.05$) increase in plasma extravasation compared to unheated skin (Figure 1). Plasma extravasation was not sig-

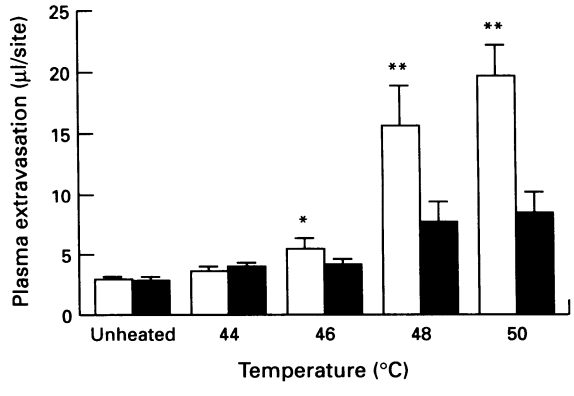

Figure 1 Extravasated plasma (µl/site) accumulated over 5 min heating at 44°C, 46°C, 48°C and 50°C (solid columns) and for 15 min afterwards (0–20 min, open columns) compared to unheated skin. Results are mean ± s.e.mean for 5 experiments, $^{**}P<0.01$ compared with unheated skin; $^{*}P<0.05$ compared with unheated skin.

nificantly increased when measured during the heating time alone (0–5 min) for any of the temperatures studied but there was a trend towards significance at 48°C and 50°C (Figure 1).

SR140333, at doses of 36 nmol kg^{-1} and above, significantly ($P<0.05$) inhibited heat-induced (5 min 48°C) plasma extravasation over 0–20 min by up to 79±3% (120 nmol kg^{-1}) (Figure 2a). $CGRP_{8-37}$ (200 nmol kg^{-1} and 400 nmol kg^{-1}) also significantly inhibited heat-induced plasma extravasation (Figure 2b). When the antagonists were co-administered (120 nmol kg^{-1} SR140333 and 200 nmol kg^{-1} $CGRP_{8-37}$) they significantly ($P<0.01$) inhibited plasma extravasation accumulated over 0–20 min by 84±15% (Figure 2c). However, this was not significantly different from extravasation observed with SR140333 alone. Both antagonists alone also significantly decreased plasma extravasation induced by a 50°C heat stimulus (5 min) by 53±10% for SR140333 (120 nmol kg^{-1}, Figure 3a) and 41±8% $CGRP_{8-37}$ (200 nmol kg^{-1}, Figure 3b).

Time-course experiments were designed to investigate plasma extravasation formed over each 5 min period 0–20 min after initiation of thermal injury. It was demonstrated that the greatest amount of plasma extravasation occurred between 5–10 min i.e. immediately after cessation of heating, but a significant ($P<0.05$) increase in plasma extravasation was observed at all time-points from 5 min. Pretreatment with SR140333 (120 nmol kg^{-1}) for 5 min led to a significant decrease in plasma extravasation accumulating over each of the 5 min periods (5–10, 10–15 and 15–20 min) (Figure 4a). In comparison $CGRP_{8-37}$ (200 nmol kg^{-1}) significantly ($P<0.05$) inhibited plasma extravasation for up to 15 min (5–10 and 10–15 min) after initiation of the thermal injury. There was a trend towards inhibition for the 15–20 min accumulation period but this did not reach significance (Figure 4b).

When [^{125}I]-HSA was injected 5, 35 or 65 min after initiation of heating and plasma extravasation allowed to accumulate for the following 30 min a significant ($P<0.01$) increase in plasma extravasation was observed (24.3±6.1, 27.5±6.0 and 36.6±8.8 µl/site for 5–35, 35–65 and 65–95 min after heat respectively compared to 4.9±0.6 µl/site for unheated skin) (Figure 5a). The administration of SR140333 (120 nmol kg^{-1}) 5 min prior to initiation of thermal injury (−5 min) caused a significant ($P<0.05$) inhibition of plasma extravasation accumulated over 5–35 min but not at the later time-points, although there was a trend towards inhibition for 35–65 min (Figure 5a). However, if SR140333 (120 nmol kg^{-1}) was administered 5 min prior to [^{125}I]-HSA injection there was a significant ($P<0.05$) inhibition of plasma extravasation accumulated over both the 5–35 and 35–65 min periods after thermal injury (Figure 5b).

In experiments to determine the selectivity of SR140333, intradermal injection of the NK_1 agonists SP and GR73632 caused a significant ($P<0.01$) increase in plasma protein extravasation compared to Tyrode-injected sites. This was further increased by co-administration with the vasodilator CGRP. Pretreatment for 5 min with SR140333, at both 120 and 360 nmol kg^{-1}, significantly ($P<0.01$) inhibited these responses. SR140333 did not inhibit plasma extravasation induced by either histamine co-injected with CGRP or bradykinin co-injected with CGRP (Figure 6).

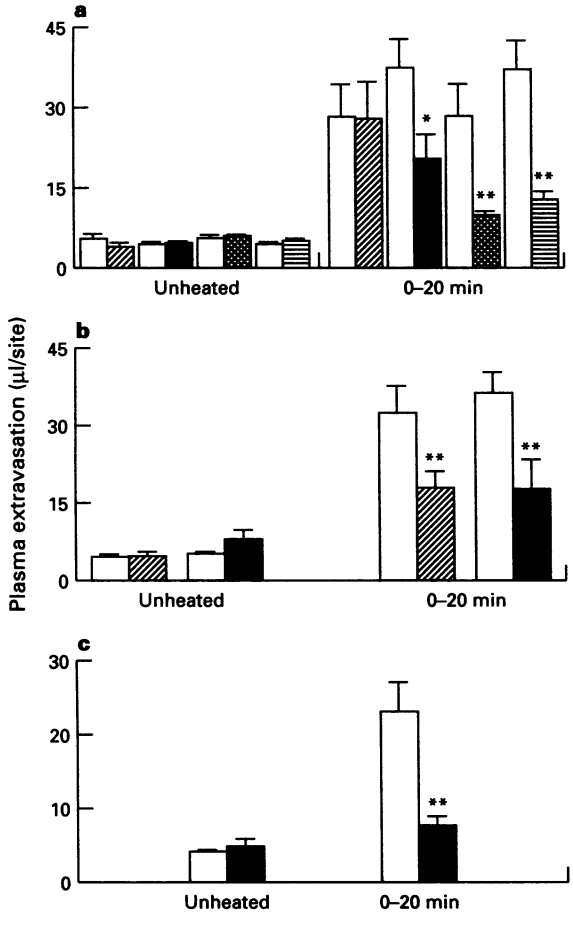

Figure 2 Extravasated plasma (µl/site) accumulated over 5 min heating at 48°C and for 15 min afterwards (0–20 min) compared to unheated skin in the presence of, (a) SR140333, 12 nmol kg^{-1} (diagonal-hatched column), 36 nmol kg^{-1} (solid column), 120 nmol kg^{-1} (cross-hatched column) and 360 nmol kg^{-1} (horizontal-hatched column); (b) $CGRP_{8-37}$ 200 nmol kg^{-1} (hatched column) and 400 nmol kg^{-1} (solid column) and (c) SR140333, 120 nmol kg^{-1} plus $CGRP_{8-37}$ 200 nmol kg^{-1} (solid column), compared to vehicle controls (open column). Results are mean±s.e.mean for 6 experiments. $^{**}P<0.01$ compared with vehicle, $^{*}P<0.05$ compared with vehicle.

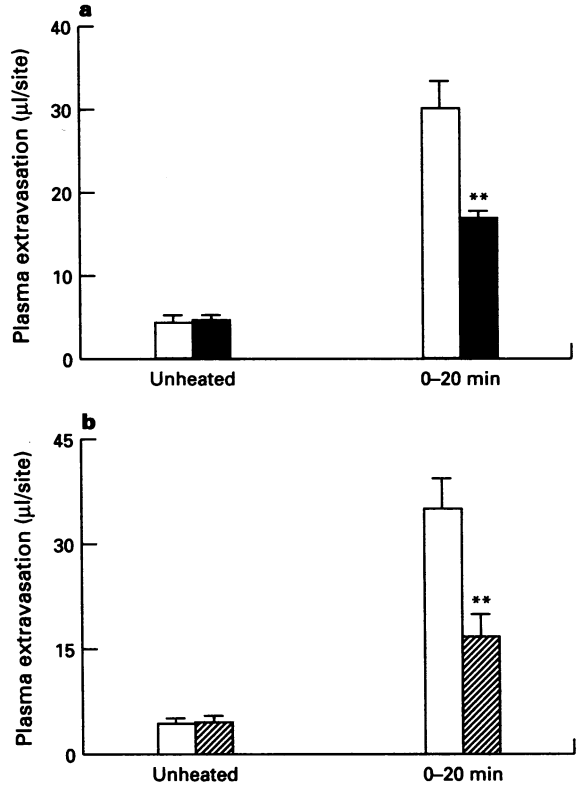

Figure 3 Extravasated plasma (µl/site) accumulated over 5 min heating at 50°C and for 15 min afterwards (0–20 min) compared to unheated skin in the presence of, (a) SR140333, 120 nmol kg^{-1} (solid column) and (b) $CGRP_{8-37}$ 200 nmol kg^{-1} (hatched column) compared to vehicle controls (open column). Results are mean± s.e.mean for 6 experiments. $^{**}P<0.01$ compared with vehicle.

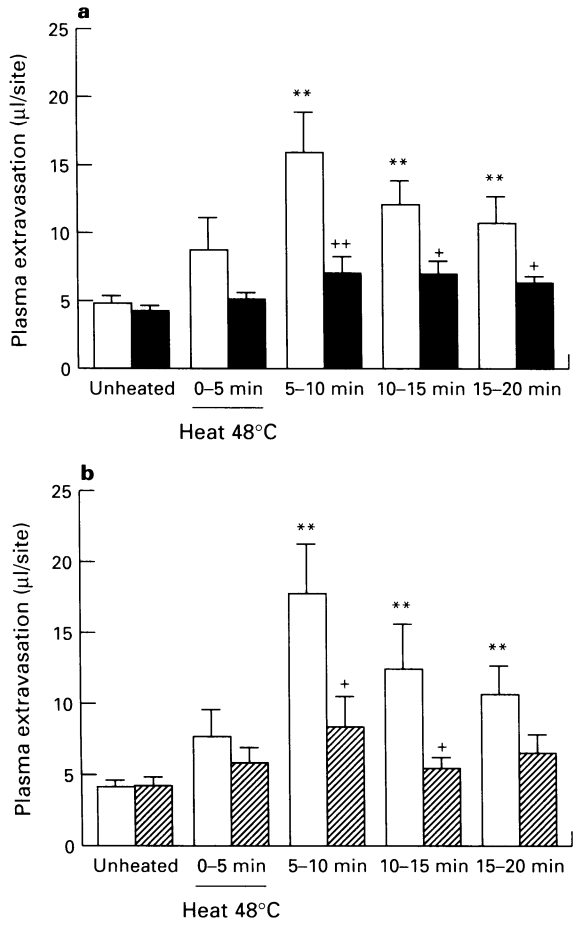

Figure 4 Extravasated plasma (µl/site) accumulated over 5 min at different times after heat (5 min for 48°C, open column) in the presence of (a) SR140333, 120 nmol kg^{-1} (solid column) and (b) $CGRP_{8-37}$ 200 nmol kg^{-1} (hatched column). Results are mean ± s.e.mean for 6 experiments. $^{**}P<0.01$ compared with unheated skin; $^{*}P<0.05$ compared with unheated skin; $^{++}P<0.01$ of vehicle; $^{+}P<0.05$ cf. vehicle.

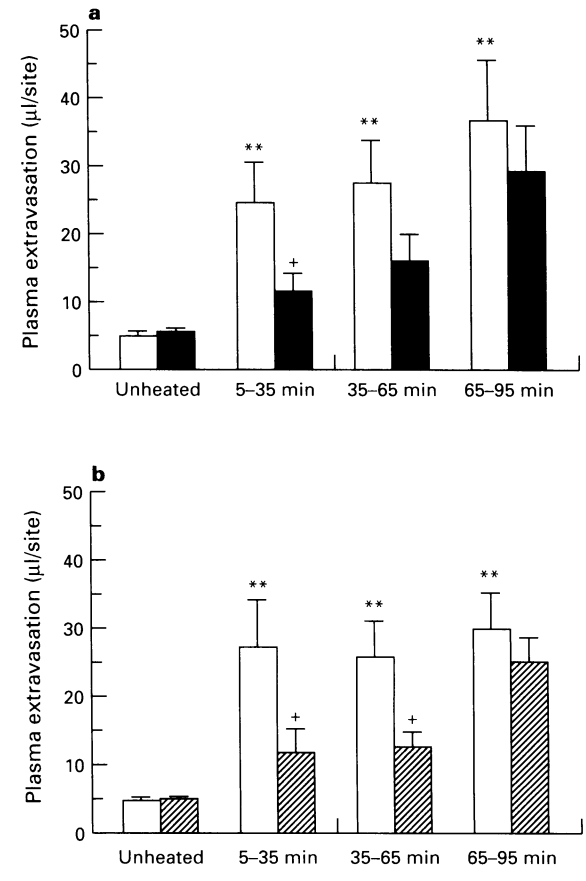

Figure 5 Effect of SR140333, 120 nmol kg^{-1} (solid or hatched column) on extravasated plasma (µl/site) accumulated over 30 min at different times after heat (5 min for 48°C) when given (a) before the heat (−5 min) or (b) at the start of the accumulation period. Vehicle controls are represented by the open columns. Results are mean ± s.e.mean for 6-7 experiments. $^{**}P<0.01$ compared with unheated skin; $^{+}P<0.05$ compared with vehicle.

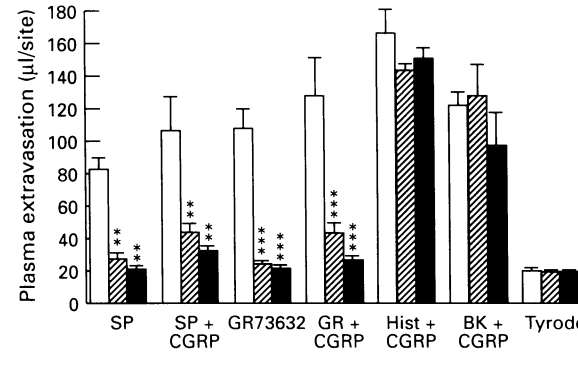

Figure 6 The effect of SR140333, 120 nmol kg^{-1} (hatched column) and 360 nmol kg^{-1} (solid column) on plasma extravasation (µl/site) induced by substance P (SP, 100 pmol/site) and GR73632 (GR, 30 pmol/site), alone and in combination with CGRP (10 pmol/site), and histamine (Hist, 10 mmol/site) and bradykinin (BK, 100 pmol/site) in combination with CGRP compared with Tyrode injected sites. Vehicle controls are represented by the open columns. Results are mean ± s.e.mean for 4 experiments. $^{**}P<0.01$ compared with vehicle, $^{***}P<0.001$ compared with vehicle.

Discussion

Previous studies have shown that SP released from sensory nerves plays a role in plasma extravasation caused by thermal injury in the rat hind paw (Saria, 1984). The present study confirms these findings in the rat dorsal skin by use of the selective, non-peptide NK_1 receptor antagonist, SR140333 (Emonds-Alt *et al.*, 1993). The doses required to cause a maximum inhibition of plasma extravasation induced after thermal injury were higher than those used by Emonds-Alt and coworkers (1993). Therefore we also carried out studies which confirm the effectiveness and selectivity of this antagonist at these doses. In our model, the endogenous agonist for the NK_1 receptor is likely to be SP because neurokinin A, another agonist for NK_1 receptors although not as potent as SP, is not released during thermal injury (Yonehara *et al.*, 1991). Using the CGRP antagonist, $CGRP_{8-37}$, at doses which are well documented and known to be selective for $CGRP_1$ receptors in rat skin (Donoso *et al.*, 1990; Gardiner *et al.*, 1990; Escott & Brain, 1993), we have demonstrated that, in addition to SP, the potent vasodilator peptide CGRP also appears to play a role in the initial plasma extravasation observed after thermal injury in rat dorsal skin. The ability of the CGRP antagonist to reduce heat-induced plasma extravasation was not unexpected as SP and CGRP are known to be co-localized in the sensory neurones of several organs including skin (Gibbons *et al.*, 1985; Lundberg *et al.*, 1985) and can act in a synergistic way to promote plasma extravasation (Brain & Williams, 1985). The lack of a dose-response effect probably indicates that this is the total contribution for CGRP in potentiating plasma protein extravasation in this model.

Interestingly, co-administration of the antagonists did not cause any significant further inhibition of plasma extravasation compared to that observed with SR140333 alone. The most

likely explanation for this is that although both mediators are vasodilators, only SP is able to increase vascular permeability. Therefore CGRP alone does not cause plasma protein extravasation but will enhance the effect of a mediator of increased permeability, such as SP (Brain & Williams, 1985). Thus inhibiting the effect of CGRP reduces plasma protein extravasation because only the effect of SP alone will be seen. However, by inhibiting the increased permeability effect of SP with SR140333, the total neurogenic effect will have been removed because CGRP cannot directly affect permeability. SR140333 reduced the plasma protein extravasation induced by intradermally-injected SP or GR73632 to that observed at Tyrode injected sites at the doses used in this study. Therefore the residual plasma protein extravasation which could not be inhibited by the antagonists is likely to be due to other mediators which are released during thermal injury; for example, bradykinin, prostaglandins and 5-hydroxytryptamine.

It has been demonstrated that capsaicin excited and then selectively desensitized C fibres (Szolcsanyi, 1977). These fibres conduct impulses characterized by a response to noxious heat from polymodal nociceptors. Furthermore, the threshold for excitation of these receptors to heat-induced pain was shown to be about 45°C (Fleischer *et al.*, 1983), a temperature similar to that which caused plasma extravasation observed in the present study. It is likely, therefore, that sensory neuropeptides are released from peripheral endings of afferent fibres after direct activation of polymodal nociceptors by heat stimulation.

Only a small increase in the amount of plasma extravasation could be measured in skin sites immediately after the cessation of heat application at both 48°C and 50°C, i.e. that accumulated over the 5 min during which heat was applied. In comparison, in the 5 min following cessation of heat (5–10 min) there was a significant ($P<0.05$) increase in plasma extravasation which was greater than that observed in the following 5 min accumulation periods (10–15 and 15–20 min). From the results of this study, it would appear that SP has a major role in the initial plasma extravasation after injury. Moreover CGRP is involved in mediating plasma extravasation for up to 15 min after the onset of thermal injury.

In the present study, it was also observed that after thermal injury induced by heat at 48°C for 5 min plasma extravasation continued for at least 95 min. This is in contrast to the findings of Saria (1984) who found no ongoing plasma extravasation after heating the hind paw skin for 5 min at various temperatures, including 48°C, although the total paw weight and skin weight were significantly increased for the 120 min time period. The difference in plasma protein extravasation observed may be due to differences in the area of the skin investigated or the methods of heating used to inflict the thermal injury. It would appear that SP has a role in the plasma extravasation formed up to 65 min after initiation of thermal injury because the presence of the NK_1 receptor antagonist at these time-points significantly inhibited plasma extravasation. However, the administration of SR140333 5 min prior to initiation of the thermal injury significantly inhibited plasma extravasation over the first 5–35 min after thermal injury, but did not significantly affect the 35–65 min accumulation period, although an inhibitory trend was observed. In comparison, when SR140333 was added 5 min prior to the accumulation period, i.e. it was present during the accumulation period only and not during the thermal injury, it significantly inhibited plasma extravasation for up to 60 min after the cessation of the thermal injury. The reason for the discrepancy in these findings is not clear; it is unlikely to be because of the duration of action of SR140333 as it has been shown to be effective for up to 4 h after administration in rats (Jung *et al.*, 1994). It was noted that the NK_1 receptor antagonist had little inhibitory effect on the oedema formation measured from 65–95 min after thermal injury. This suggests that neurokinins are not the major mediators increasing permeability during the later period. It will be of interest to determine in future studies the mechanisms involved in this later phase of oedema formation.

In conclusion the results from the present study confirm that SP is likely to play a role in the initial plasma extravasation after the thermal injury and demonstrate for the first time that CGRP may also play a role in the acute response. In addition, the results suggest that SP may play a role in the ongoing plasma extravasation which occurs for up to 65 min after initiation of thermal injury.

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