Involvement of tachykinins in plasma extravasation induced by bradykinin and low pH medium in the guinea-pig conjunctiva

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1 The effect of bradykinin, capsaicin, substance P and low pH medium on plasma extravasation in the guinea-pig conjunctiva has been studied. Evans blue dye was measured in the conjunctiva after local instillation of the agents into the conjunctival sac.

2 Bradykinin (2–50 nmol), capsaicin (20–50 nmol) and substance P (0.5–5 nmol) caused a dose-dependent increase in plasma extravasation with the following order of potency: substance P > bradykinin = capsaicin. The effect of capsaicin (50 nmol) and substance P (5 nmol) was abolished by the tachykinin NK₁ receptor antagonist, CP-99,994 (8 µmol kg⁻¹, i.v.) ($P < 0.01$), whereas CP-100,263 (8 µmol kg⁻¹, i.v.) the inactive enantiomer of CP-99,994 was without effect. CP-99,994 inhibited by 70% ($P < 0.01$) the effect of bradykinin.

3 The kinin B₂ receptor antagonist, Hoe 140 (icatibant, 10 nmol kg⁻¹, i.v.) abolished the response to bradykinin (50 nmol) ($P < 0.01$), but did not affect the responses to capsaicin (50 nmol) or substance P (5 nmol). Plasma extravasation induced by low pH medium (pH 1) was abolished by CP-99,994 ($P < 0.01$) and by Hoe 140 ($P < 0.01$).

4 The present findings suggest that: endogenous or exogenous tachykinins increase plasma extravasation in the guinea-pig conjunctiva by activation of NK₁ receptors; bradykinin-induced plasma extravasation is mediated by tachykinin release from sensory nerve endings; low pH media cause plasma extravasation via release of kinins that by activation of B₂ receptors release tachykinins from sensory nerve endings.

Keywords: Neurogenic inflammation; conjunctiva; substance P; capsaicin; tachykinin NK₁ receptors; bradykinin; kinin B₂ receptors; low pH

Introduction

A subpopulation of primary sensory neurones is characterized by the ability to release neuropeptides from their peripheral endings, thus causing a series of effects collectively referred to as neurogenic inflammation. In most tissues these effects consist of arterial vasodilatation, leakage of plasma proteins and recruitment of inflammatory cells. The neuropeptides released from primary sensory neurones include calcitonin gene-related peptide (CGRP) and the tachykinins, substance P and neurokinin A. CGRP mediates vasodilatation in certain tissues and organs, whereas the tachykinins increase plasma protein extravasation and promote leukocyte adhesion to the vascular endothelium (Holzer, 1988; Maggi *et al.*, 1989). These effects of tachykinins are mediated, with a few exceptions (Tousignant *et al.*, 1993), by activation of tachykinin NK₁ receptors (Eglezos *et al.*, 1992), probably located on the endothelial cells of postcapillary venules (Bowden *et al.*, 1994).

The conjunctiva is innervated by peptide-containing sensory nerves (Elsas *et al.*, 1994). These nerves are sensitive to the excitatory action of capsaicin, the pungent principle contained in the plants of the genus *Capsicum*. Early studies by N. Jancso and coworkers reported that capsaicin or other congeners of capsaicin cause oedema or interendothelial gap formation in the venules in the conjunctiva of the rat and guinea-pig (Jancso, 1955; Jancso *et al.*, 1968). More recently, Saria and coworkers (1983) showed that a variety of exogenous or endogenous agents, including the proinflammatory peptide, bradykinin, induce plasma extravasation in rodent conjunctiva. The kinins, bradykinin and kallidin, are formed during injury and inflammation from plasma or tissue pre-

cursor, respectively (Regoli & Barabe, 1980). Kinins acting directly on effector cells or indirectly by releasing other inflammatory mediators increase and sustain the inflammatory response.

The aim of the present paper was to investigate the mechanism and possible pathophysiological implications of the bradykinin-induced plasma extravasation in the guinea-pig conjunctiva. Because in other tissues the increase in plasma extravasation induced by bradykinin is mediated by activation of a neurogenic inflammatory mechanism (Lundberg & Saria, 1983; Ichinose *et al.*, 1990; Bertrand *et al.*, 1993a; Geppetti, 1993), this possibility was explored in the guinea-pig conjunctiva. To determine whether kinin B₂ receptors mediate the response to bradykinin the peptide B₂ receptor antagonist, Hoe 140 (icatibant) (Wirth *et al.*, 1991) was used. To determine whether the action of kinin and capsaicin was due to tachykinin release, the NK₁ receptor antagonist, CP-99,994 (McLean *et al.*, 1993) was used. Finally, the involvement of kinin formation and of neurogenic inflammatory mechanisms in the plasma extravasation induced by acid media was investigated.

Methods

Animals and experimental procedure

Male albino Hartley guinea-pigs (Rodentia, Bergamo, Italy) were used in this study. They were kept in a temperature-controlled environment with standard laboratory food and water freely available. The animals (body weight, 350–450 g) were anaesthetized with sodium pentobarbitone (45 mg kg⁻¹, i.p.) and the jugular vein was cannulated. Deep anaesthesia

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was monitored throughout the experiment and additional anaesthetic administered as required. Evans blue dye (3% solution in 0.9% NaCl; Sigma, St. Louis, MO, U.S.A.) was used to measure plasma extravasation, which was injected (30 mg kg^{-1} , i.v. over 5 s) via the jugular vein. Immediately after Evans blue administration, agents were given by local instillation (dissolved in $10 \mu\text{l}$ of saline) into the conjunctival sac by means of a micropipette. The solution was applied uniformly throughout the conjunctival surface. One single conjunctiva, chosen at random, was treated with the stimulus in each animal. Local application of drugs or agents to the conjunctiva did not cause any adverse reaction of any aversive behavioural response.

Terminal anaesthesia (pentobarbitone, 40 mg kg^{-1} , i.v.) was always administered 30 s before terminating the experiments. At this time it is assumed that the increase in vascular permeability is already completed. Experiments were terminated by opening of the chest 5 min after injection of the tracer, inserting a cannula into the ascending aorta through the left ventricle, and perfusing the circulation for 2 min with phosphate buffer (pH 5; Sigma Chemical, St. Louis, MO) at a pressure of 130 mmHg. The conjunctiva adherent to either the palpebra or the sclera was removed. Tissues were blotted on paper and weighed. All tissues were incubated in 1 ml of formamide (Merk, Darmstadt, Germany) at 60°C for 18 h to

extract the extravasated Evans blue dye (Lundberg & Saria, 1983).

Capsaicin (50 nmol) was dissolved in a solution containing 10% ethanol, 5% Tween 80 and 85% 0.9% NaCl. Further dilutions were in 0.9% NaCl. All the other drugs were dissolved in 0.9% NaCl. CP-99,994 and CP-100,263 were given 5 min before the stimulus, and Hoe 140 15 min before the stimulus by i.v. administration through the jugular vein. Low pH media were obtained by adding HCl to the saline solution until the desired pH was obtained.

Measurement of plasma extravasation

The extravasation of Evans blue dye-labelled macromolecules from the microcirculation in the tissue was quantified by measuring the optical density of the formamide extracts at a wavelength of 620 nm with a spectrophotometer (Model UV1204, Shimadzu Scientific Instruments, Inc., Japan). The amount of Evans blue dye extravasated in the tissues, expressed in ng mg^{-1} of wet weight, was interpolated from a standard curve of Evans blue concentrations (0.1 to $5 \mu\text{g ml}^{-1}$).

Drugs

CP-99,994 (+)-(2S,3S)-3-(2-methoxybenzyl-amino)-2-phenylpiperidine) and CP-100,263 (–)-(2R,3R)-3-(2-methoxybenzyl-amino)-2-phenylpiperidine) were kind gifts from Dr J.A. Lowe III (Pfizer Inc., Groton, CT, U.S.A.), and Hoe 140 (D-Arg-[Hyp³, Thi⁵, D-Tic⁷, Oic⁸]-bradykinin) was a kind gift from Dr K.J. Wirth (Hoechst, Germany). Substance P and bradykinin were purchased from Peninsula Laboratories, Inc. (Merseyside, UK); capsaicin was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Statistical analysis

All data are expressed as mean $\pm$ s.e.mean. Mean values of spectrophotometric measurements of Evans blue dye extravasation were analysed by one-way analysis of variance. Comparisons between means in each condition were performed by Bonferroni's multiple range test. Student's *t* test for paired or unpaired data was used when applicable. Differences of $P < 0.05$ were considered significant.

Results

Effect of bradykinin, capsaicin and substance P

Baseline Evans blue dye extravasation in untreated conjunctiva of guinea-pigs was $11.2 \pm 1.3 \text{ ng mg}^{-1}$ ($n = 6$). Instillation of $10 \mu\text{l}$ of saline into the conjunctiva contralateral to the untreated side did not increase plasma extravasation significantly ($12.4 \pm 1.1 \text{ ng mg}^{-1}$, $n = 6$). Instillation of bradykinin and substance P caused a dose-dependent increase in Evans blue dye extravasation (Figure 1). The vehicle of 50 nmol capsaicin (10% ethanol, 5% Tween 80 and 85% 0.9% NaCl) did not induce any increase in the extravasation of the Evans blue dye ($9.7 \pm 1.1 \text{ ng ml}^{-1}$, $n = 5$) as compared with the effect of saline ($10.9 \pm 1.6 \text{ ng ml}^{-1}$, $n = 5$) applied to the contralateral eye. Capsaicin caused a dose-dependent increase in plasma extravasation (Figure 1). Substance P was approximately 10 times more potent in increasing the extravasation of the Evans blue dye in the conjunctiva than capsaicin and bradykinin. Bradykinin and capsaicin were virtually equipotent.

Effect of tachykinin and kinin receptor antagonists

Both CP-99,994 ($8 \mu\text{mol kg}^{-1}$, i.v.) and CP-100,263 ($8 \mu\text{mol kg}^{-1}$, i.v.), the inactive enantiomer of CP-99,994 did not affect baseline Evans blue dye extravasation (data not

Figure 1 Effect of increasing doses of substance P, bradykinin and capsaicin (hatched columns) on the Evans blue dye extravasation in the conjunctiva of guinea-pigs. Drugs were applied in a volume of $10 \mu\text{l}$ to the conjunctiva. Open columns indicate the Evans blue dye extravasation produced by the vehicle of each drug ($10 \mu\text{l}$). Columns are mean $\pm$ s.e.mean of at least 4 experiments.

shown). Pretreatment with CP-99,994 ($8 \mu\text{mol kg}^{-1}$, i.v.) abolished the response to substance P (5 nmol), whereas pretreatment with $8 \mu\text{mol kg}^{-1}$ of CP-100,263 (i.v.), did not affect the response to substance P (Figure 2). Pretreatment with CP-99,994 ($8 \mu\text{mol kg}^{-1}$, i.v.) abolished the increase in Evans blue dye extravasation induced by capsaicin (50 nmol) and reduced by about 70% the response to bradykinin (50 nmol) (Figure 2).

Hoe 140 (10 nmol kg^{-1} , i.v.) did not affect basal plasma extravasation in the guinea-pig conjunctiva (data not shown). Pretreatment with Hoe 140 abolished the Evans blue dye extravasation caused by bradykinin (50 nmol), but did not affect the response induced by substance P (5 nmol) (Figure 3).

Effect of low pH media

Application of low pH media ($10 \mu\text{l}$) to the conjunctival surface increased Evans blue dye extravasation in a manner inversely related to the pH of the medium. Media at pH higher than 2 did not affect plasma extravasation. A medium at pH 1.5 increased Evans blue dye extravasation to $24.3 \pm 3.5 \text{ ng mg}^{-1}$ ($n = 4$). Medium at pH 1 caused an increase in Evans blue dye extravasation ($43.7 \pm 6.8 \text{ ng mg}^{-1}$, $n = 5$),

Figure 2 Effect of pretreatment with the tachykinin NK₁ receptor antagonist, CP-99,994 ($8 \mu\text{mol kg}^{-1}$, i.v.), or its enantiomer CP-100,263 ($8 \mu\text{mol kg}^{-1}$, i.v.), on the Evans blue dye extravasation evoked by the instillation of substance P (5 nmol), capsaicin (50 nmol) or bradykinin (50 nmol) in the guinea-pig conjunctiva. Open column indicates the Evans blue dye extravasation induced by 0.9% saline ($10 \mu\text{l}$). Drugs were applied in a volume of $10 \mu\text{l}$ to the conjunctiva. Columns are mean $\pm$ s.e.mean of at least 4 experiments. * $P < 0.01$ versus vehicle.

Figure 3 Effect of pretreatment with the kinin B₂ receptor antagonist, Hoe 140 (10 nmol kg^{-1} , i.v.), on the Evans blue dye extravasation evoked by the instillation of substance P (5 nmol) or bradykinin (50 nmol) in the guinea-pig conjunctiva. Open column indicates the Evans blue dye extravasation induced by 0.9% saline ($10 \mu\text{l}$). Drugs were applied in a volume of $10 \mu\text{l}$ to the conjunctiva. Columns are mean $\pm$ s.e.mean of at least 4 experiments. * $P < 0.01$ versus vehicle.

that was comparable to that induced by submaximal doses of substance P or bradykinin (Figure 4), without causing any macroscopical damage to the conjunctiva. Media at pH 0.5 or lower caused macroscopical damage of the conjunctiva and therefore were not further investigated. Pretreatment with CP-99,994 ($8 \mu\text{mol kg}^{-1}$, i.v.) or pretreatment with Hoe 140 (10 nmol kg^{-1} , i.v.) abolished the extravasation induced by pH 1 medium (Figure 4).

Discussion

The stimuli used in the present investigation were all able to cause plasma extravasation in the guinea-pig conjunctiva. Substance P, capsaicin and bradykinin induced a dose-dependent increase in plasma extravasation. Low pH media increased plasma extravasation in a manner inversely related to the pH value of the medium. Plasma extravasation evoked by substance P was completely abolished by the tachykinin NK₁ receptor antagonist, CP-99,994. This compound exhibits a lesser calcium antagonist property than the parent molecule CP-96,345 (McLean *et al.*, 1993). In addition, the observation that CP-100,263, the enantiomer of CP-99,994, which is inactive on tachykinin NK₁ receptors, did not show any effect on the substance P-induced response indicates that CP-99,994 acted selectively to inhibit the NK₁ receptor. Therefore, the present data suggest that the plasma extravasation caused by exogenously applied substance P to the guinea-pig conjunctiva is entirely mediated by activation of NK₁ receptors. Capsaicin excites sensory nerves causing the release of tachykinins and CGRP from the peripheral endings of these nerves. The finding that CP-99,994 blocked the plasma extravasation induced by capsaicin suggests that the plasma extravasation mediated by the release of endogenous tachykinins is also due to the activation of NK₁ receptors. This conclusion is in agreement with recent data reporting that plasma extravasation caused by capsaicin i.v. in the guinea-pig conjunctiva was abolished by the tachykinin NK₁ receptor antagonist, RPR 100893 (Lee *et al.*, 1994). CGRP is released with tachykinins from sensory nerve endings, and regulates blood flow in various organs of different species. The present findings, do not exclude the possibility that CGRP by regulating conjunctival blood flow may modulate plasma extravasation induced by stimulation of sensory nerves. However, they exclude the possibility that CGRP

Figure 4 Effect of pretreatment with the tachykinin NK₁ receptor antagonist, CP-99,994 ($8 \mu\text{mol kg}^{-1}$, i.v.), and the kinin B₂ receptor antagonist, Hoe 140 (10 nmol kg^{-1} , i.v.), on the Evans blue dye extravasation evoked by the instillation of a medium at pH 1 in the guinea-pig conjunctiva. Open column indicates the Evans blue dye extravasation induced by 0.9% saline ($10 \mu\text{l}$). Drugs were applied in a volume of $10 \mu\text{l}$ to the conjunctiva. Columns are mean $\pm$ s.e.mean of at least 4 experiments. * $P < 0.01$ versus vehicle.

plays a direct role in neurogenic plasma extravasation in the conjunctiva.

The effects of bradykinin are mediated by stimulation of B₁ and B₂ receptors (Regoli & Barabe 1980). Since the selective kinin B₂ receptor antagonist, Hoe 140 (Wirth *et al.*, 1991) abolished plasma extravasation induced by locally applied bradykinin, it is concluded that this effect of bradykinin is mediated by activation of B₂ receptors. This antagonist did not affect substance P-induced plasma extravasation, which indicates that kinin release is not involved in the plasma extravasation evoked by substance P in the guinea-pig conjunctiva.

Bradykinin stimulates inflammatory responses, including plasma extravasation, via its own receptors on effector cells or indirectly by releasing mediators from intermediate cells. Bradykinin-induced plasma extravasation in the guinea-pig conjunctiva is markedly inhibited by CP-99,994, thus indicating that NK₁ receptor activation is involved in this response. Bradykinin stimulates sensory nerves and causes the release of sensory neuropeptides from peripheral endings of these neurones (Geppetti, 1993). It is proposed that local application of bradykinin to the guinea-pig conjunctiva increases the leakage of plasma proteins mainly by a neurogenic inflammatory mechanism. CP-99,994, at a dose that completely blocked substance P- and capsaicin-induced plasma extravasation, significantly inhibited, but did not abolish the response caused by bradykinin. It is probable that the CP-99,994-resistant component of the bradykinin-induced response is mediated by the activation of bradykinin B₂ receptors on the venular endothelium. A dual mechanism, in large part neurogenic, and in minor part non-neurogenic for inducing plasma extravasation by locally applied bradykinin has also been shown in other organs and tissues including the trachea (Lundberg & Saria, 1983) and the nasal mucosa (Bertrand *et al.*, 1993a).

The pH of the medium during tissue injury, ischaemia or inflammation may fall to values around 5 or 4, and hyperaemia may lower the pH of the lacrimal fluid. In addition, acid solution may accidentally come in contact with the conjunctival surface. The observation that CP-99,994 blocked plasma extravasation evoked by low pH medium suggests that in the guinea-pig conjunctiva, protons increase plasma extravasation by releasing tachykinins from sensory nerve endings. Recently, protons have been shown to activate a cation conductance and to release neuropeptides selectively in capsaicin-sensitive primary sensory neurones (Geppetti *et al.*, 1990; Bevan & Yeats, 1991). Hence, at least part of the

proinflammatory action of hydrogen ions may derive from their ability to excite sensory nerves and cause neurogenic inflammation. Evidence obtained in dorsal root ganglion neurones in culture or in slices of peripheral tissues suggests protons may have a direct action on sensory nerves where they activate the channel non selective for cations opened by capsaicin (Geppetti *et al.*, 1991; Liu & Simon, 1994). However, the present data showing that Hoe 140 abolished the plasma extravasation induced by low pH medium in the conjunctiva raise the possibility that kinins play an initial role in mediating the inflammatory response to protons.

Since both kinin and tachykinin receptor antagonists blocked the plasma extravasation evoked by low pH media, the hypothesis may be advanced that these two classes of peptide mediators do not act independently, but rather utilize a common final pathway. The observation that Hoe 140 did not affect the plasma extravasation evoked by substance P, whereas CP-99,994 reduced markedly the response to bradykinin suggests that protons promote the following cascade of events: low pH medium releases kinins which by stimulating sensory neuropeptide release increase plasma extravasation. Examples of kinins formed following different types of inflammatory insult, and causing inflammatory responses through the release of sensory neuropeptides have been reported recently (Bertrand *et al.*, 1993b; Ahluwalia *et al.*, 1994; Ricciardolo *et al.*, 1994; Yoshihara *et al.*, 1994). There is also pharmacological evidence that the increase in mucosal blood flow of the gastric wall induced by acid back diffusion is in part mediated by kinins (Holzer & Petho, 1994). Mucosal hyperaemia induced by acid back diffusion is mediated by sensory nerve stimulation (Holzer, 1992), thus suggesting that a mechanism similar to that reported here for the conjunctiva may also be present in the stomach.

Conjunctivitis may derive from several types of insult. Here, we report that certain stimuli cause plasma extravasation in the conjunctiva by a neurogenic inflammatory mechanism. We also show that a pathway involving kinin generation and the subsequent release of sensory neuropeptides plays a major role in the inflammatory response to low pH media. Hence, drugs which inhibit kinin B₂ and tachykinin NK₁ receptors may be of value in the treatment of conjunctivitis.

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