Br. J. Pharmacol. (1994), **112**, 453–460

Mediation by bradykinin of rat paw oedema induced by collagenase from *Clostridium histolyticum*

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1 Collagenases are thought to play a major role in the pathology of gas gangrene caused by *Clostridium histolyticum*, because they can destroy the connective tissue barriers. We investigated possible mediators involved in the oedema formation and plasma protein extravasation which follow the injection of a collagenase (EC 3.4.24.3) from *Clostridium histolyticum* into one hind paw of anaesthetized rats.

2 The magnitude of the oedema following a subplantar injection was dependent on the dose of collagenase (30, 100 and 300 μg) injected. It reached its maximum within 30 min and remained unchanged for at least 5 h. Plasma protein extravasation into the paw was most pronounced within 20 min of the injection. Heat-inactivated collagenase was ineffective.

3 The B_2 bradykinin (BK) antagonist icatibant (D-Arg-[Hyp^3-Thi^5-D-Tic^7-Oic^8] bradykinin, formerly named Hoe-140) reduced oedema formation in a dose-dependent manner with a maximal reduction of around 65% at a dose of 100 nmol kg^{-1} (s.c.). A significant effect could already be observed at a dose of 10 nmol kg^{-1}. The duration of the effect of icatibant (100 nmol kg^{-1}) was found to be at least 3 h. These results demonstrate the high potency and long duration of action of icatibant. Pretreatment of rats with the bradykinin B_1 antagonist, des-Arg^9-[Leu^8]-BK did not affect collagenase-induced paw oedema. Thus, the observed collagenase-induced effects are mainly mediated by BK through activation of B_2 receptors.

4 Pretreatment of adult rats with capsaicin (125 mg kg^{-1}, s.c.) three weeks before the collagenase injection caused a significant attenuation of the paw oedema and of plasma extravasation but was significantly less effective than icatibant (100 nmol kg^{-1}, s.c.). The non-peptide substance P antagonist, CP-96,345 (10 μmol kg^{-1}, i.v.) significantly reduced collagenase-induced oedema formation to a degree comparable with that seen after capsaicin pretreatment. The inhibition by the substance P antagonist was significantly smaller than that seen after icatibant. The inhibitory effect of icatibant in capsaicin-pretreated rats, or of icatibant together with CP-96,345 in untreated rats, was not greater than that of icatibant alone in rats treated with the vehicle for either capsaicin or CP-96,345. CP-96,344 (10 μmol kg^{-1}, i.v.), the inactive enantiomer of CP-96,345, did not affect collagenase-induced paw oedema. In capsaicin-pretreated rats, CP-96,345 (10 μmol kg^{-1}, i.v.) did not reduce collagenase-induced paw oedema.

The subplantar injection of bradykinin (30 nmol) induced a paw oedema comparable with that induced by collagenase (100 μg). CP-96,345 (10 μmol kg^{-1}, i.v.), but not CP-96,344 (10 μmol kg^{-1}, i.v.), significantly reduced the bradykinin-induced paw oedema. These findings indicate that collagenase leads to the release of bradykinin; bradykinin then stimulates afferent C-fibre terminals and causes the release of substance P and probably also neurokinin A, which augment the oedema-inducing effect of bradykinin.

5 Indomethacin or mepyramine plus cimetidine failed to inhibit collagenase-induced paw oedema. Thus, prostaglandins and histamine do not seem to be involved in collagenase-induced paw oedema.

6 After subplantar injection of collagenase, the sensitivity scores in a modified formalin-test rapidly increased during the first 10 min. This increase was abolished by pretreatment with icatibant (100 nmol kg^{-1}, s.c.) indicating that the stimulation of nociceptive afferent neurones following injection of collagenase is due to the action of released kinins.

7 In conclusion, bradykinin appears to be the main mediator of inflammation induced by a collagenase from *Clostridium histolyticum*. As well as having direct relevance to a known pathological condition, collagenase-induced paw oedema could prove to be a useful model in inflammation research and in the investigation of bradykinin antagonists. The present results might provide an experimental basis for clinical investigations of the effects of icatibant in infectious diseases where the release of collagenases from bacteria causes rapid spreading of inflammation.

Keywords: Collagenase; *Clostridium histolyticum*; bradykinin; icatibant; CP-96,345; capsaicin; paw oedema; plasma protein extravasation

Introduction

Clostridium histolyticum is one of the bacteria that are found in clostridial myonecrosis, i.e. gas gangrene. This life-threatening disease is caused by toxins produced by clostridial bacteria. Such bacteria are present throughout the environment. Although the majority of cases occur after injuries such as open fractures, wounds, or burns, clostridial myonecrosis has also been reported in cases of 'clean' surgery (Parker, 1969). The management of patients with gas gangrene is difficult and requires intensive and aggressive medical intervention including extensive resection of necrotic tissue or even amputation to save the patient's life. The earliest signs of gas gangrene are disproportionate pain and pronounced oedema at the site of the wound. These symptoms spread as the Clostridia invade neighbouring tissue.

Collagenases, which are components of the β-toxin produced by *C. histolyticum*, are thought to play a major role in

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the pathology of gas gangrene because they destroy the connective tissue barriers and thereby facilitate the invasion of the tissue by bacteria (Hatheway, 1990). It has been reported that anti-inflammatory drugs such as aspirin and dexamethasone inhibited to some degree collagenase-induced inflammation (Vargaftig *et al.*, 1976). The aim of the present study was to elucidate further which mediators are involved in the oedema and plasma protein extravasation following a subplantar injection in rats of collagenase (EC 3.4.24.3) from *C. histolyticum*.

Some of these results have been presented to the British Pharmacological Society (Legat *et al.*, 1993).

Methods

Female Sprague Dawley rats (200–280 g) were anaesthetized with pentobarbitone sodium (50 mg kg^{-1}, i.p.). Collagenase (EC 3.4.24.3) from *C. histolyticum*, 100 μg dissolved in 100 μl of a 154 mM NaCl solution (saline), was given, as a subplantar injection (s.p.), into one hind paw. In experiments in which the time course of development of paw oedema after the injection of different doses of collagenase was studied, 30 or 300 μg doses of the enzyme were also used. In all experiments, the contralateral hind paw served as control and was injected with 100 μl saline.

The magnitude of the oedema was assesssed by measuring the paw volume with a plethysmometer (Ugo Basile, Italy) before, and at given time intervals during, the first hour after the collagenase injection; in some experiments it was also measured 300 min after the injection.

For the quantitative evaluation of plasma protein extravasation, the extravasation of Evans blue dye into the paws was measured (Gamse *et al.*, 1980). Evans blue (20 mg kg^{-1}) was injected i.v. 5 min before the subplantar injection of collagenase. At the end of the experiment, the rats were exsanguinated, each hind paw was cut off at the ankle joint and immersed in 5 ml formamide for 24 h at 50°C. The concentration of Evans blue in the formamide was determined photometrically at 620 nm.

The time course of plasma protein extravasation after the subplantar injection of collagenase into one hind paw and of saline into the contralateral hind paw was studied by measuring the Evans blue extravasation in the paws during 4 consecutive 10 min periods following the collagenase injection. A separate group of rats was used for each of the 4 consecutive 10 min periods. In the first group of rats, Evans blue was injected at the same time as the collagenase; in the second, third and fourth groups of rats Evans blue was injected 10, 20 and 30 min after the collagenase, respectively. In each group of rats, the hind paws were detached 10 min after the Evans blue injection and Evans blue extravasation determined as described above.

Oedema formation and plasma extravasation in the hind paw was also measured after the subplantar injection of heat-inactivated collagenase (exposure to 60°C for 30 min).

In another set of experiments, oedema formation was measured after a subplantar injection of bradykinin (30 nmol in 100 μl saline).

Bradykinin (BK) antagonists

Icatibant In order to study the effectiveness of this bradykinin B_2 antagonist, rats were given a s.c. injection of 1, 3, 10, 30, 100, 300 or 1000 nmol kg^{-1} icatibant, 60 min before a subplantar injection of 100 μg collagenase and the paw volume was measured during the 60 min period following the collagenase injection. The duration of action of icatibant was measured by injecting rats with 100 nmol kg^{-1} icatibant 10, 30, 60, 180, 360 or 540 min before the subplantar injection of collagenase (100 μg).

Des-Arg^9-[Leu^8]-bradykinin Rats received i.v. injections of this bradykinin B_1 antagonist (200 nmol kg^{-1}) 10 min before the subplantar injection of 100 μg collagenase.

The ensuing paw oedema was measured after both bradykinin antagonists.

Capsaicin pretreatment

Adult rats were treated with capsaicin three weeks before the collagenase experiments according to a regimen similar to that used by Esplugues *et al.* (1989). Capsaicin, 25 mg kg^{-1}, was injected into rats, under ether anaesthesia, twice on day one and in the morning of day two; in the afternoon of day two the dose used was 50 mg kg^{-1}. Before the morning injections of capsaicin, the rats were given a mixture of terbutaline (0.2 mg kg^{-1}), aminophylline (20 mg kg^{-1}) and atropine (0.1 mg kg^{-1}) i.p. to prevent bronchoconstriction by capsaicin. An equal number of rats was treated similarly, but received vehicle instead of capsaicin. One group of capsaicin-pretreated rats and one group of vehicle-pretreated rats received icatibant (100 nmol kg^{-1}, s.c.), 60 min before the collagenase injection. The corresponding control groups received a s.c. injection of 1 ml kg^{-1} saline. Paw volume was measured during the 60 min following the subplantar injection of collagenase (100 μg) and plasma extravasation was determined at the end of the experiment.

The non-peptide substance P antagonist, CP-96,345

Rats were pretreated either with CP-96,345 (10 μmol kg^{-1}, i.v.) 10 min prior to the injection of collagenase, or with icatibant (100 nmol kg^{-1}, s.c.) 60 min before collagenase. In another group of rats both pretreatments were combined. Control rats received the vehicles by the corresponding routes. Paw volume was measured during the 60 min period following the collagenase injection and plasma protein extravasation was determined at the end of the experiment.

In order to verify that the effects of CP-96,345 were specific, control experiments were carried out in rats which were injected i.v. 10 min before collagenase with the inactive enantiomer, CP-96,344 (10 μmol kg^{-1}).

Pretreatment of rats with capsaicin combined with CP-96,345

Rats were pretreated with capsaicin as described above. Three weeks later they were injected with CP-96,345 (10 μmol kg^{-1}, i.v.) or saline 10 min before the subplantar injection of collagenase. The magnitude of the oedema was determined as above.

Bradykinin-induced paw oedema: pretreatment with CP-96,345 or CP-96,344

Paw oedema was induced by a subplantar injection of bradykinin (30 nmol in 100 μl saline). Rats were pretreated with CP-96,345 (10 μmol kg^{-1}, i.v.), CP-96,344 (10 μmol kg^{-1}, i.v.) or saline (i.v.) 10 min before the subplantar injection of bradykinin.

Blockade of histamine receptors or inhibition of prostaglandin formation

Mepyramine (35 μmol kg^{-1}) and cimetidine (40 μmol kg^{-1}) were injected i.p. 60 min before collagenase (100 μg); indomethacin (30 μmol kg^{-1}) was injected i.p. 60 min before collagenase (100 μg). The corresponding control rats received the appropriate vehicles. Paw volume was measured either during the 60 min period (mepyramine plus cimetidine) or the 300 min period (indomethacin) following the collagenase injection.

Activity scores

The behavioural reactions of rats after subplantar injection of collagenase (100 μg in 100 μl saline) were rated in a modified formalin-test (Cohen *et al.*, 1984) by sensitivity scores (0 = walking or sitting normally; 1 = walking or sitting favouring the paw not injected with collagenase; 2 = lifting the injected paw off the ground; 3 = licking the injected paw). The integrated sensitivity scores for consecutive 5 min periods after the injection of collagenase were calculated for each rat; the total observation period after the subplantar injection was 60 min. The integrated sensitivity score was calculated by summing the score values of each second during a 5 min period and dividing the resulting sum by 300.

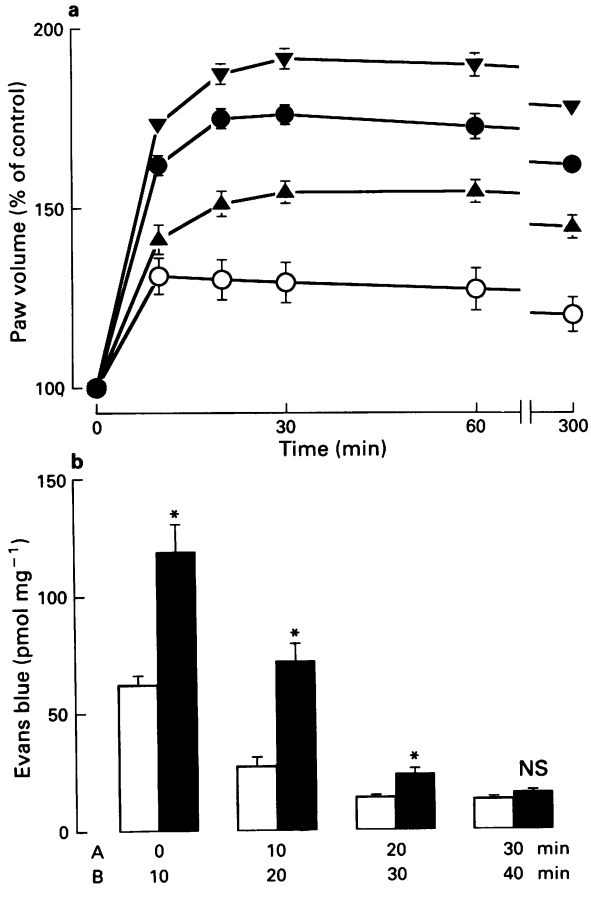

Figure 1 Collagenase-induced paw oedema. (a) Time course and dose-response relationship of collagenase-induced paw oedema. Collagenase (30 ▲, 100 ●, 300 μg ▼) or saline (○) was administered as a subplantar injection (100 μl) into rat hind paws at time 0 min. Paw volume was monitored during the following 300 min. Volume of paw oedema is expressed as a percentage of the paw volume before the subplantar injection of collagenase or saline. Data are means ± s.e.mean; ($n = 6$). (b) Collagenase-induced extravasation of plasma proteins as indicated by the Evans blue content of the paws in 4 consecutive 10 min periods following the subplantar injection of collagenase (100 μg in 100 μl saline) at time 0. The contralateral hind paws were injected with 100 μl saline. The plasma protein extravasation during each of the 4 consecutive 10 min periods after collagenase injection was evaluated by using a separate group of rats for each 10 min period. 'Time A' gives the time after the injection of collagenase at which the rats in the different groups were injected with Evans blue (20 mg kg^{-1}, i.v.). 'Time B' gives the time after the injection of collagenase at which the hind paws of rats in the different groups were detached for the determination of their Evans blue content. Data are means ± s.e.mean of the Evans blue extravasation, given as pmol mg^{-1} wet weight, in the paws injected with collagenase (solid columns) and the contralateral paws injected with saline (open columns). Significance of difference from saline control: *$P < 0.05$, NS = not significant ($P < 0.10$). $n = 6$.

Sixty min before the subplantar injection of collagenase, rats were treated either with icatibant (100 nmol kg^{-1}, s.c.) or with saline.

Materials

Collagenase (EC 3.4.24.3) from *Clostridium histolyticum* (Type II, Sigma), Evans blue, aminophylline and des-Arg^9-[Leu^8]-bradykinin (Sigma, St. Louis, MO, U.S.A.); icatibant (D-Arg-[Hyp^3-Thi^5-D-Tic^7-Oic^8-bradykinin] formerly named Hoe-140, mol. wt. = 1863) (Hoechst AG, Frankfurt/Main, Germany); CP-96,345 {(2S,3S)-*cis*-2-(diphenylmethyl)-*N*-((2-methoxyphenyl)-methyl)-1-azabicyclo[2.2.2]octan-3-amine dihydrochloride; mol. wt. = 499.02} CP-96,344 {[2R,3R)-*cis*-2-(diphenylmethyl)-*N*-((2-methoxyphenyl)-methyl)-1-azabicyclo-[2.2.2]octan-3-amine dihydrochloride; mol. wt. = 499.02} (Pfizer Inc., Groton, Conn., U.S.A.); mepyramine and cimetidine (Smith Kline Beecham, U.K.); indomethacin and atropine sulphate (Merck Sharp & Dohme, U.S.A.); pentobarbitone sodium (Sanofi Santé, France); capsaicin (Fluka, Buchs, Switzerland); terbutaline sulphate (Astra, Wedel/Holstein, Germany); ethanol and Tween 80 (Merck, Darmstadt, Germany); bradykinin (mol. wt. = 1306.45) (Bachem, Bubendorf, Switzerland).

Capsaicin was dissolved in a mixture of 10% (v/v) ethanol and 10% (v/v) Tween 80 in saline.

Statistical analysis

The paw volumes measured after the subplantar injections of collagenase, bradykinin or of saline were expressed as a % of the value determined immediately prior to the injection. From the values obtained at regular intervals during the course of the experiment the area under the curve was calculated. The data obtained for paw volume and for Evans blue extravasation were tested for deviation from normality by the Shapiro-Wilk test (Conover, 1980). In order to test for a possible heteroskedasticity, the Levene test (Sachs, 1984) was used. Multiple comparisons between the treatment groups were then made using the least significance difference test (Sachs, 1984). The mean sensitivity scores for each consecutive 5 min period obtained after the subplantar injection of collagenase were compared with the values for saline-injected control rats using the Mann-Whitney U test. In the experiments in which the time course of plasma protein extravasation after subplantar injection of collagenase was studied, the extravasation in paws injected with collagenase was compared with that in the contralateral paws injected with saline using the Wilcoxon matched pairs signed rank test. Multiple nonparametric comparisons with a control were used to compare the net amount of plasma protein which leaked out of paw vessels in the 2nd, 3rd and 4th 10 min period with that in the 1st 10 min period after the collagenase injection.

Results

Time course of development of paw oedema after injection of three different doses of collagenase

Subplantar injection of collagenase (30, 100 and 300 μg in 100 μl saline) into rat hind paws induced a dose-dependent increase in paw volume (Figure 1a). The paw oedema reached its maximum within 30 min; it was only slightly smaller after 300 min. The collagenase-injected paws were significantly larger ($P < 0.001$) than the control paws injected with 100 μl saline. Subplantar injection of heat-inactivated collagenase (100 μg) did not induce paw oedema or plasma protein extravasation. Paw volume was increased to 119 ± 3% after 30 min and 120 ± 3% after 60 min in paws injected with saline, and to 120 ± 3% after 30 min and 121 ± 4% after 60 min in paws injected with heat-inactivated col-

lagenase ($n = 6$). Extravasation of Evans blue was 59 ± 11 pmol mg^{-1} wet weight in paws injected with saline and 68 ± 11 pmol mg^{-1} wet weight in paws injected with heat-inactivated collagenase ($n = 6$).

Time course of plasma protein extravasation after collagenase injection

The amount of plasma protein which leaked out of the paw vessels after the injection of collagenase (100 μg, s.p.) was largest during the first 10 min and, thereafter, declined with time. In the contralateral hind paws injected with saline, the extravasation of plasma proteins was also largest during the 1st 10 min and then declined. In the 1st, 2nd and 3rd 10 min period, the extravasation of plasma proteins in paws injected with collagenase was significantly ($P<0.05$) larger than in the contralateral paws injected with saline; in the 4th 10 min period the difference was not significant ($P<0.10$) (Figure 1b).

The net amount of plasma protein extravasated, i.e. the difference between the values for collagenase-injected and for saline-injected paws, was similar in the 1st and 2nd 10 min periods but was significantly smaller in the 3rd and 4th observation periods ($P<0.05$, $P<0.001$, respectively).

Effects of bradykinin antagonists on collagenase-induced oedema

Icatibant: dose-response relationship Icatibant, 10–1000 nmol kg^{-1}, given s.c. 60 min before a subplantar injection of collagenase (100 μg), significantly ($P<0.01$) reduced the resulting paw oedema. The inhibition caused by doses of 30–1000 nmol kg^{-1} icatibant was not significantly different from that caused by 10 nmol kg^{-1} (Figure 2a).

Icatibant: duration of action Icatibant (100 nmol kg^{-1}, s.c.) given up to 3 h before the subplantar injection of collagenase significantly ($P<0.01$) reduced collagenase-induced paw oedema (Figure 2b). Icatibant did not reduce the oedema when administered 6 or 9 h before collagenase.

Icatibant: effect on plasma protein extravasation Icatibant (100 nmol kg^{-1}, s.c.) given 60 min before a subplantar injection of collagenase (100 μg) caused a reduction in plasma extravasation that was similar to the reduction in the paw oedema (Figure 3b and Figure 4b).

Des-Arg^9-[Leu^8]-bradykinin Pretreatment of rats with the bradykinin B_1 antagonist des-Arg^9-[Leu^8]-BK (200 nmol kg^{-1}, i.v.) 10 min before a subplantar injection of collagenase did not affect collagenase-induced paw oedema (Table 1).

Effects of capsaicin on collagenase-induced paw oedema

Pretreatment of adult rats with capsaicin (125 mg kg^{-1}, s.c. three weeks before collagenase) significantly ($P<0.05$) reduced the collagenase-induced paw oedema (Figure 3a). However, the reduction brought about by capsaicin was significantly ($P<0.05$) smaller than that caused by icatibant (100 nmol kg^{-1}, s.c.). The inhibitory effect of icatibant in capsaicin-pretreated rats was not different from that in control rats.

The extravasation of Evans blue induced by collagenase was significantly reduced by capsaicin pretreatment ($P<0.001$), but to a significantly ($P<0.05$) lesser degree than by icatibant. The inhibitory effects of icatibant and capsaicin were not additive (Figure 3b).

Effect of the substance P antagonist, CP-96,345, on collagenase-induced oedema

CP-96,345 (10 μmol kg^{-1}, i.v.) significantly ($P<0.05$) reduced the collagenase-induced paw oedema (Table 1) to a degree

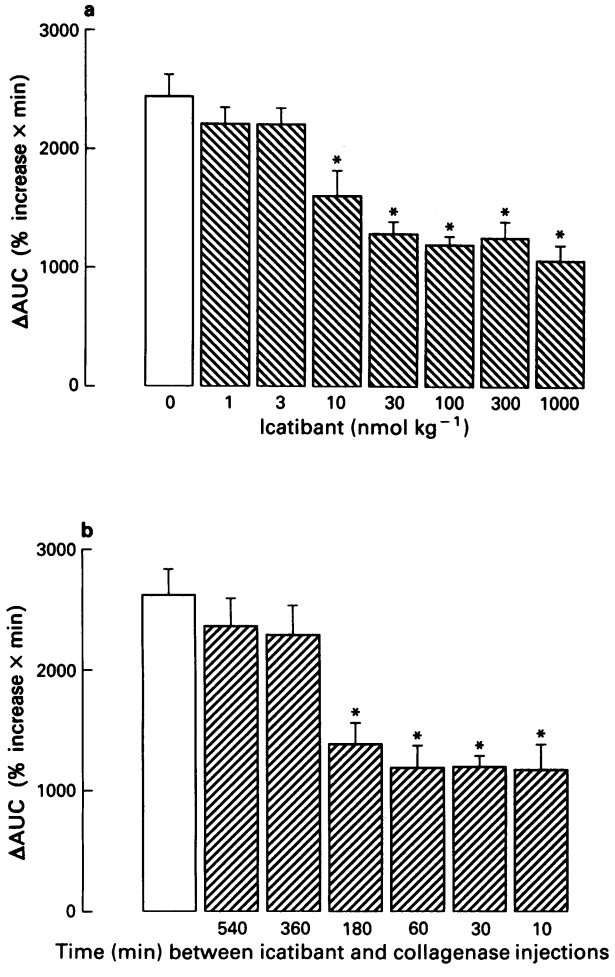

Figure 2 Effect of icatibant on collagenase-induced paw oedema. The area under the volume-time curve (AUC) of paw volume increase above the pre-injection volume was calculated for paws injected with collagenase and for the contralateral paws injected with saline. The difference between the two AUC values gives the net increase in paw volume induced by collagenase. (a) Effect of increasing doses of icatibant on collagenase-induced paw oedema. Icatibant (1, 3, 10, 30, 100, 300, or 1000 nmol kg^{-1} in 1 ml kg^{-1} saline, s.c.) was injected 60 min before collagenase (hatched columns). Control rats received the corresponding volume of saline s.c. (open column). Paw volume was measured repeatedly for 60 min after the injection of collagenase (100 μg) into one hind paw and saline (100 μl) into the contralateral paw. Data are means ± s.e.mean of the net increase of paw volume (ΔAUC) after injection of collagenase. Significance of difference from saline control: $^*P<0.01$; $n = 6$. (b) Effect of increasing the pretreatment interval on the inhibitory effect of icatibant. Icatibant (100 nmol kg^{-1}, s.c.) was injected 10, 30, 60, 180, 360 or 540 min before the subplantar injection of collagenase (100 μg) or saline (100 μl) into the rat hind paws. Data are means ± s.e.mean of the ΔAUCs (net increase of paw volume) after injection of collagenase. Significance of difference from saline control: $^*P<0.01$; $n = 6$.

similar to that caused by capsaicin pretreatment, but was significantly ($P<0.05$) less effective than icatibant (100 nmol kg^{-1}, s.c.). The effects of icatibant and CP-96,345 were not additive (Figure 4a). In capsaicin-pretreated rats CP-96,345 (10 μmol kg^{-1}, i.v.) did not further reduce collagenase-induced paw oedema (data not shown).

In contrast to icatibant, CP-96,345 (10 μmol kg^{-1}) did not significantly reduce collagenase-induced plasma extravasation, nor did it augment the effect of icatibant on plasma extravasation (Figure 4b).

CP-96,344, the inactive enantiomer of CP-96,345, did not affect collagenase-induced paw oedema (Table 1).

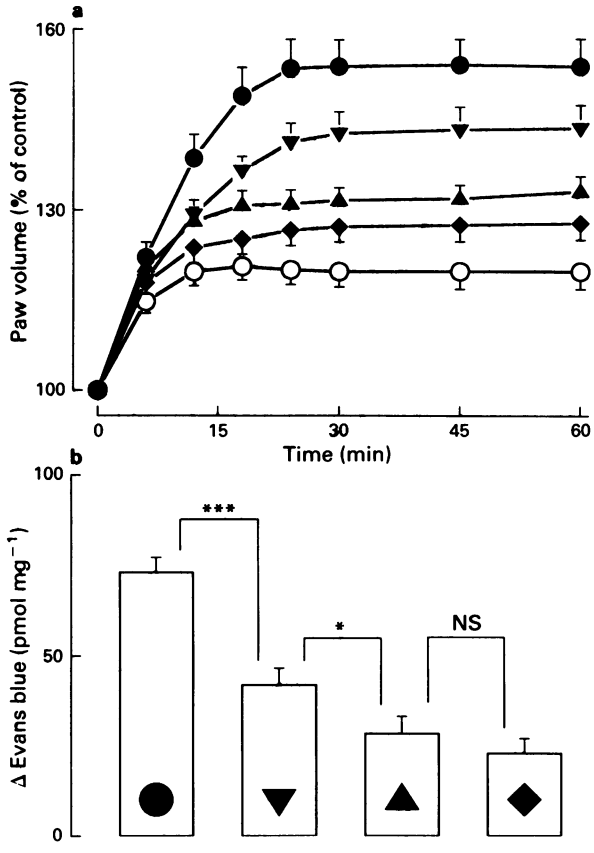

Figure 3 Effect of capsaicin on collagenase-induced paw oedema and plasma extravasation. Paw oedema was induced by subplantar injection of collagenase (●, 100 μg). The contralateral hind paws were injected with 100 μl saline (○). Rats were pretreated either with capsaicin (▼, 125 mg kg^{-1}, s.c., 3 weeks prior to the collagenase injection), or with icatibant (▲, 100 nmol kg^{-1}, s.c., 60 min before collagenase injection) or with capsaicin together with icatibant (◆). (a) Effect of capsaicin and/or icatibant on collagenase-induced paw oedema. Paw volume was measured for 60 min after subplantar injection of collagenase or saline. The values given are means ± s.e.mean ($n = 6$) of paw volumes expressed as a percentage of the pre-injection paw volume. For reasons of clarity only the paw volume-time curve of saline-injected paws of non pretreated control rats is given. This was not different from the curve obtained with pretreated rats ($n = 6$). (b) Effect of capsaicin and/or icatibant on collagenase-induced extravasation of plasma proteins. Evans blue (20 mg kg^{-1}) was injected i.v. 5 min before the subplantar injection of collagenase or saline. Immediately after the last measurement of paw volume, i.e. 60 min after collagenase injection, rats were exsanguinated, the hind paws cut off and the Evans blue content determined. Column heights are means ± s.e.mean of the difference (Δ) between Evans blue extravasation in paws injected with collagenase and Evans blue extravasated in the contralateral paws injected with saline. The amount of Evans blue extravasated in paws injected with saline was about 50 pmol mg^{-1} wet weight in each group of rats. Significance of differences between groups: $^{*}P < 0.05$, $^{***}P < 0.001$; NS, not significant. $n = 6$.

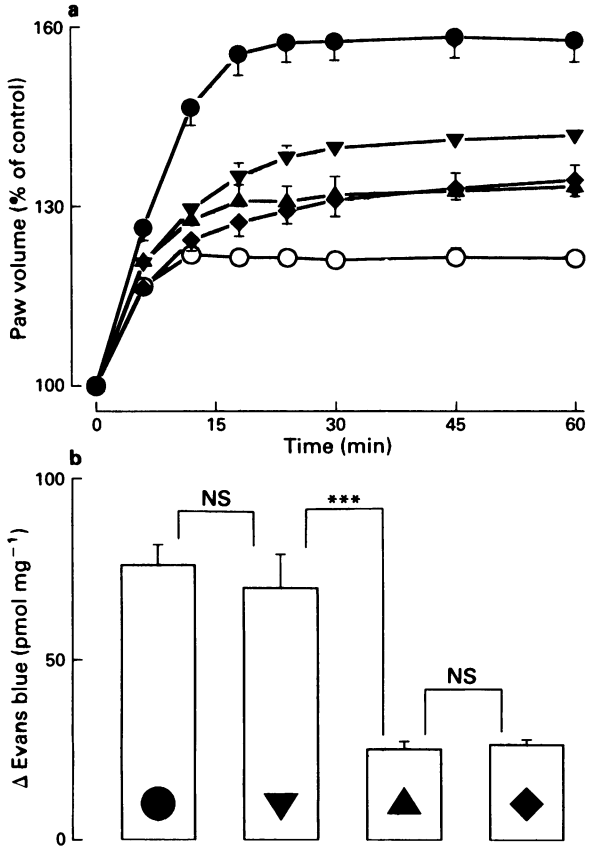

Figure 4 Effect of pretreatment with CP-96,345 on collagenase-induced paw oedema and plasma extravasation. Paw oedema was induced by subplantar injection of collagenase (●, 100 μg), the contralateral hindpaws were injected with 100 μl saline (○). Rats were pretreated with CP-96,345 (▼, 10 μmol kg^{-1}, i.v., 10 min before collagenase), or with icatibant (▲, 100 nmol kg^{-1}, s.c., 60 min before collagenase) or with both CP-96,345 and icatibant (◆). (a) Effect of the substance P antagonist, CP-96,345 and/or icatibant on collagenase-induced paw oedema. Paw volume was measured during the 60 min period after subplantar injection of collagenase or saline. The values given are means ± s.e.mean ($n = 6$–11) of paw volumes expressed as a percentage of the pre-injection paw volume. For reasons of clarity only the paw volume-time curve of saline-injected paws of control rats is given; it is similar to the curve obtained for the saline injected paws of drug-pretreated rats. (b) Effect of substance P antagonist, CP-96,345 and/or icatibant on collagenase-induced extravasation of plasma proteins. Evans blue (20 mg kg^{-1}) was injected i.v. 5 min before the subplantar injection of collagenase or saline. Sixty min after the collagenase injection the rats were exsanguinated, the hind paws cut off and the Evans blue content determined. Column heights represent means ± s.e.mean of the difference (Δ) between Evans blue extravasation in paws injected with collagenase and Evans blue extravasation in the contralateral paws injected with saline. The amount of Evans blue extravasated in paws injected with saline was about 50 pmol mg^{-1} wet weight in each group of rats. Significance of difference between treatments: $^{***}P < 0.001$; NS, not significant. $n = 6$–11.

Effect of CP-96,345 or CP-96,344 on bradykinin-induced paw oedema

Subplantar injection of BK (30 nmol) caused a paw oedema comparable with that caused by collagenase (100 μg, s.p.), i.e. about 160% of the pre-injection paw volume.

Pretreatment of rats with the substance P antagonist, CP-96,345 (10 μmol kg^{-1}, i.v.) significantly reduced both paw oedema induced by bradykinin (30 nmol) (Figure 5) and collagenase-induced paw oedema to a similar extent, i.e. to about 140% of the pre-injection paw volume.

The inactive enantiomer CP-96,344 had no effect on bradykinin-induced oedema formation (Figure 5).

Effects of drugs that block histamine receptors or inhibit prostaglandin formation

The pretreatment of rats with mepyramine (35 μmol kg^{-1}, i.p.) combined with cimetidine (40 μmol kg^{-1}, i.p.) did not reduce collagenase-induced paw oedema (Table 1). Indomethacin (30 μmol kg^{-1}, i.p.) also had no effect on collagenase-induced paw oedema (Table 1).

Behaviour after subplantar injection of collagenase

Immediately after subplantar injection of collagenase into the hind paws of saline-pretreated rats, there was a rapid in-

Table 1 Effect of drugs, which block histamine or substance P receptors or prostaglandin formation, on collagenase-induced oedema in the hind paw of the rat

Drug	Pretreatment Dose (μmol kg^{-1})	Route	% increase in paw volume Time after collagenase 30 min	60 min	300 min
			%	%	%
des-Arg^9[Leu^8]-BK	0.2	i.v.	178 ± 6	178 ± 4	–
Saline			175 ± 3	178 ± 3	–
Mepyramine	35	i.p.			
+ cimetidine	40	i.p.	177 ± 4	172 ± 3	–
Saline			179 ± 5	175 ± 4	–
Indomethacin	30	i.p.	182 ± 2	182 ± 1	176 ± 2
Saline			181 ± 3	181 ± 3	177 ± 3
CP-96,345	10	i.v.	140 ± 1*	142 ± 1*	–
Saline			157 ± 3	158 ± 4	–
CP-96,344	10	i.v.	171 ± 3	174 ± 3	–
Saline			170 ± 2	168 ± 3	–

The dose of collagenase given by subplantar injection was 100 μg (dissolved in 100 μl saline). Results are expressed as a percentage of paw volume before collagenase injection. Data are means ± s.e.mean. $n = 6-9$. Significance of difference from saline control: *$P < 0.05$.

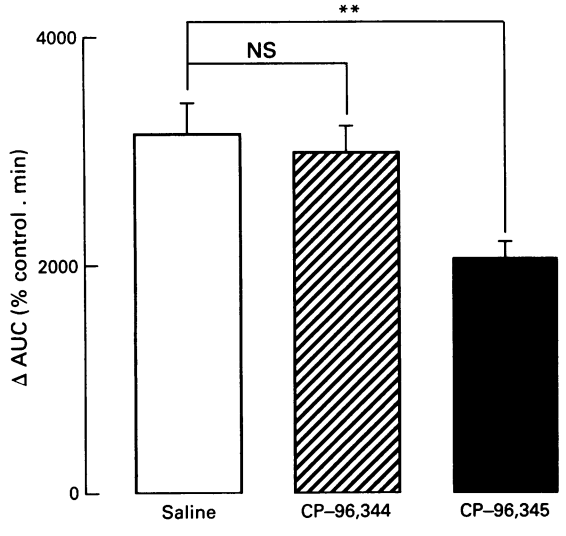

Figure 5 Effect of pretreatment with CP-96,345 or CP-96,344 on bradykinin-induced paw oedema. The area under the paw volume-time curve (AUC) above the level of pre-injection volume was calculated for paws injected with bradykinin (BK, 30 nmol in 100 μl saline) and for the contralateral paws injected with 100 μl saline. The difference between the two AUC values gives the net increase in paw volume induced by BK. Rats were pretreated either with CP-96,345 (10 μmol kg^{-1}, i.v., 10 min before BK; solid column) or with CP-96,344 (10 μmol kg^{-1}, i.v., 10 min before BK; hatched column). Control rats received the corresponding volume of saline i.v. (open column). Paw volume was measured repeatedly for 60 min after the injection of BK into one hind paw and after the injection of saline into the contralateral hind paw. Data are means ± s.e.mean of the net increase of paw volume (ΔAUC) after the injection of BK. Significance of difference from saline control: **$P < 0.01$; NS, not significant. $n = 10$.

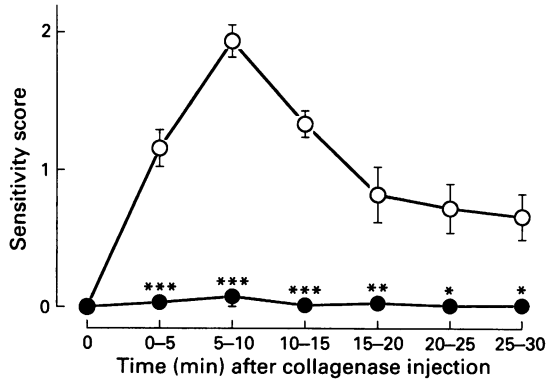

Figure 6 Sensitivity scores after subplantar injection of collagenase. Behavioural reactions of rats which received a subplantar injection of collagenase (100 μg) were rated with sensitivity scores from 0 to a maximum of 3. Sixty min before the collagenase injection, rats were pretreated s.c. either with icatibant (●, 100 nmol kg^{-1}) or with a similar volume of saline (○). The total observation period following the injection of collagenase was 60 min. For reasons of clarity only the first 30 min after the collagenase injection are given in the graph; thereafter, until the end of the observation period, the difference in the sensitivity scores for rats pretreated with icatibant and for control rats remained unchanged (*$P < 0.02$). Values are means ± s.e.mean of the integrated sensitivity scores in consecutive 5 min periods after the injection of collagenase. The integrated sensitivity score in a 5 min period was calculated by summing the sensitivity scores for each second during the 5 min period and dividing the sum by 300. Significance of difference between control and icatibant-pretreated rats: *$P < 0.02$; **$P < 0.01$; ***$P < 0.002$. $n = 6-9$.

crease in the sensitivity scores (Figure 6). A peak in the integrated sensitivity score was reached in the second 5 min period after the injection of collagenase. Thereafter, the score declined rapidly. Pretreatment of rats with icatibant (100 nmol kg^{-1}, s.c.) 60 min before collagenase almost completely ($P < 0.002$) prevented the effect of collagenase.

Discussion

The β-toxin of *Clostridium histolyticum* is comprised of a mixture of at least seven collagenases, which differ from mammalian collagenases in their mode of action (Hatheway, 1990). Clostridial collagenases act on all known types of collagen and can cleave native triple helical collagen into small fragments, mostly tripeptides, whereas mammalian collagenases are specific for certain types of collagen and often cleave only one protein bond in a polypeptide chain of triple helical collagen, resulting in two polypeptide fragments (Han *et al.*, 1992). Thus, clostridial collagenases are very effective in destroying the connective tissue barrier. Breakdown of connective tissue facilitates bacterial invasion into tissues and provides conditions necessary for bacterial proliferation and growth.

The subplantar injection of collagenase from *C. his-*

tolyticum into rat hind paws induced a dose-dependent paw oedema. Injection of heat-inactivated collagenase did not cause oedema, as has previously been shown by Vargaftig *et al.* (1976), and also did not cause extravasation of plasma proteins. Paw oedema development and plasma protein extravasation were most pronounced during the first 20 min after subplantar injection of collagenase and in most experiments the paw oedema reached its maximum within 30 min. The time course of sensitivity scores showed a peak in the 2nd 5 min period after the challenge with collagenase. The rapid onset of the effects of clostridial collagenase implies that the collagenase itself and/or the immediate release of fast acting inflammatory mediators, rather than breakdown of the tissues, leads to an increase in vascular permeability and to stimulation of sensory nerves. This contrasts with the report by Vargaftig *et al.* (1976) who suggested that the early phase of paw oedema development after injection of clostridial collagenase into rat hind paws is only of minor importance for the inflammatory action of this enzyme.

Pretreatment of rats with a potent bradykinin B_2 receptor antagonist, icatibant [formerly named compound I (Lembeck *et al.*, 1991) or Hoe-140 (Hock *et al.*, 1991; Wirth *et al.*, 1991)], caused a pronounced reduction of the collagenase-induced paw oedema and of plasma protein extravasation. In addition, icatibant abolished the increase in sensitivity scores in a behaviour test after subplantar injection of collagenase in unanaesthetized rats.

Icatibant has been shown to be inactive against a great variety of mediators including angiotensin II, noradrenaline, histamine, acetylcholine, 5-hydroxytryptamine, substance P and neurokinin A (Lembeck *et al.*, 1991; Wirth *et al.*, 1991; Damas & Remacle-Volon, 1992; Rhaleb *et al.*, 1992). Since icatibant does not inhibit the action of des-Arg^9-BK at the B_1 receptor (Hock *et al.*, 1991; Rhaleb *et al.*, 1992), the present results point towards an involvement of B_2, but not B_1 receptors in collagenase-induced paw oedema.

Pretreatment of adult rats with capsaicin in a dose sufficient to impair the function of sensory afferent nerve fibres (Gamse *et al.*, 1980; 1981) significantly decreased the collagenase-induced paw oedema and the plasma protein extravasation. A significant reduction of the paw oedema, similar to that caused by capsaicin, was also induced by CP-96,345, a non-peptide antagonist of substance P which is specific for NK_1-receptors (McLean *et al.*, 1991; Snider *et al.*, 1991). The reduction of the collagenase-induced paw oedema brought about by icatibant was, however, significantly greater than that caused by capsaicin or by CP-96,345. The effect of pretreatment with icatibant together with capsaicin or icatibant together with CP-96,345 did not exceed that of icatibant alone. These results suggest that the collagenase-induced paw oedema is caused firstly by a direct action of BK and secondly by the action of neuropeptides released from sensory afferent neurones by BK. The ability of BK to release neuropeptides from peripheral terminals of capsaicin-sensitive primary afferents has recently been reviewed by Geppetti (1993). In addition, our findings indicate that, in collagenase-induced paw oedema, the effects of neuropeptides released from sensory afferent neurones are mainly mediated by activation of NK_1-receptors. Thus, tachykinins, especially substance P and probably also neurokinin A, seem to be the most important of the neuropeptides involved in the inflammatory effect of collagenase. The assumption that bradykinin releases tachykinins which then act on NK_1-receptors is further supported by our findings that CP-96,345 not only significantly reduced the collagenase-induced paw oedema but also caused a similar reduction of rat paw oedema induced by an equipotent dose of BK. Nonspecific effects of CP-96,345, reported to appear when it is used in high doses (Constantine *et al.*, 1991; Donnerer *et al.*, 1992; Griesbacher *et al.*, 1992; Lembeck *et al.*, 1992a), can be ruled out since the inactive enantiomer, CP-96,344, did not affect the oedema induced by collagenase or BK. In addition, CP-96,345 did not affect collagenase-induced paw oedema in rats which had been pretreated with capsaicin. This indicates that CP-96,345 blocks tachykinins released from capsaicin-sensitive afferent neurones.

Whereas CP-96,345 significantly reduced collagenase-induced formation of paw oedema, no significant reduction of collagenase-induced plasma protein extravasation by CP-96,345 was observed. At present there is no satisfactory explanation for this discrepancy. It may indicate differences in the mechanisms which lead to the increases in the vascular permeabilities for protein-free fluid and for plasma proteins.

It has been reported previously (Lembeck *et al.*, 1992b) that infusion of BK into the femoral artery of rat isolated hind legs does not cause histamine release. In the present study, a role for histamine in the formation of the paw oedema induced by clostridial collagenase was excluded since it was not affected by pretreatment with mepyramine and cimetidine. These findings are in accordance with observations of Vargaftig *et al.* (1976). However, we could not confirm a reduction of the collagenase-induced paw oedema by indomethacin. Our findings thus suggest that prostaglandins are not involved in collagenase-induced paw oedema.

The part of the paw oedema which is prevented by icatibant but is not affected by CP-96,345 or capsaicin is probably mediated by a direct action of BK on endothelial cells and/or other parts of the blood vessel walls. This action could involve vasodilatation of pre-capillary arterioles, constriction of post-capillary venules, and increase of microvascular leakage as a result of endothelial cell contraction and widening of intracellular junctions (Johnson, 1979).

The BK-induced increase in vascular permeability after injection of collagenase is mediated through activation of bradykinin B_2 receptors, since the bradykinin B_1 antagonist des-Arg^9-[Leu^8]-BK had no effect on the formation of the oedema. This also applies to the action of BK on sensory afferent neurones leading to the peripheral release of tachykinins.

From the time course of the formation of the collagenase-induced paw oedema, it can be seen that the inhibitory effects of icatibant, capsaicin and CP-96,345 are present during the first minutes of the oedema formation. This indicates that BK and tachykinins are released very shortly after the subplantar injection of collagenase and that later than 30 min after the injection of collagenase no further mediator with a noticable impact on oedema formation is released.

Icatibant was found to be a potent agent for counteracting the collagenase-induced paw oedema and plasma protein extravasation. However, icatibant was not able to prevent these effects completely. In addition to the oedema, haemorrhages were observed in the paws injected with collagenase, but not in the paws injected with saline. This has already been described by Vargaftig *et al.* (1976). Just *et al.* (1970) reported that the ability of collagenase to cause haemorrhage is due to a direct collagenolytic action on the basement membranes of small vessels, thereby destroying the integrity of the vessel walls. They also showed that BK, by itself, is not able to induce haemorrhage. Thus, destruction of vessel walls leading to bleeding might account for that part of collagenase-induced paw oedema which could not be blocked by treatment with icatibant. However, the involvement of other, as yet undetected, inflammatory mediators cannot be excluded.

In conclusion, collagenase from *Clostridium histolyticum* causes severe paw oedema and plasma protein extravasation. Bradykinin, acting on bradykinin B_2 receptors, appears to be the main mediator of this inflammatory response. Part of the effect of BK is due to the release of substance P, and possibly also neurokinin A, from the peripheral terminals of sensory afferent neurones. Histamine and prostaglandins are apparently not involved in the inflammatory action of collagenase.

Icatibant could provide a new direction for supportive therapy in the prevention of tissue destruction and bacterial invasion in infectious diseases where the release of col-

lagenase from bacteria causes rapid spread of tissue inflammation.

The authors thank Mrs E. Painsipp for expert technical assistance. The work was supported by grants from the Austrian Academy of Sciences and from the Franz-Lanyar-Stiftung.

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(Received July 23, 1993
Revised January 31, 1994
Accepted February 9, 1994)