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Involvement of tissue kallikrein but not plasma kallikrein in the development of symptoms mediated by endogenous kinins in acute pancreatitis in rats

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1 In order to investigate the mechanism of kinin release leading to vascular symptoms in acute interstitial-oedematous pancreatitis, the novel, selective inhibitors of tissue kallikrein, (2*S*,2′*R*)-2-(2′-amino-3′-(4′-chlorophenyl)propanoylamino)-*N*-(3-guanidinopropyl)-3-(1-naphthyl)propanoamide (FE999024, CH-2856), and of plasma kallikrein, (2′*S*,2″*R*)-4-(2′-(2″(carboxymethylamino)-3″-cyclohexyl-propanoylamino)-3′-phenyl-propanoylamino)piperidine-1-carboxamidin (FE999026, CH-4215), were used in experimental caerulein-induced pancreatitis in rats.

2 Oedema formation and plasma protein extravasation during the 2 h infusion of caerulein were inhibited in a dose-dependent manner by i.p. pretreatment with FE999024 (7–60 μmol kg^{-1}) while FE999026 had no effect at the same doses.

3 Haemoconcentration and hypovolaemia associated with the pancreatic oedema formation during pancreatitis were significantly attenuated by FE999024 at a dose of 20 μmol kg^{-1}. The reduction in circulating plasma volume was not affected by FE999026.

4 Accumulation of amylase and lipase in the pancreas was dose-dependently reduced by FE999024 while enzyme activities in the blood serum were increased by FE999024 at 60 μmol kg^{-1} indicating improved enzyme removal from the tissue. Enzyme activities in the tissue and in the blood remained unaffected by FE999026.

5 FE999024 (20 μmol kg^{-1}) largely inhibited increased tissue kallikrein-like activity in the pancreas during acute pancreatitis and also strongly attenuated influx of plasma kallikrein into the tissue. FE999026 (20 μmol kg^{-1}) significantly inhibited plasma kallikrein-like activity in the pancreas but had no effect on tissue kallikrein-like activity.

6 In conclusion, vascular kinin-mediated symptoms observed during oedematous pancreatitis in the rat are caused by the action of tissue kallikrein in the pancreas whereas an involvement of plasma kallikrein seems to be unlikely.

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Abbreviations: p-KK, plasma kallikrein; t-KK, tissue kallikrein

Introduction

Ever since the discovery of the kallikrein-kinin system it had been speculated that the release of kinins could play an important role in the pathophysiology of acute pancreatitis (Forrell, 1955; Werle *et al*., 1958). Previous investigations using the bradykinin B_2 receptor antagonist, icatibant (Hoe-140), have demonstrated that kinins are not involved in the induction of the disease, but play a pivotal role in the development of the vascular inflammatory symptoms, i.e. oedema formation and plasma protein extravasation, and their associated phenomena such as hypotension, haemoconcentration, hypovolaemia and retention of activated digestive enzymes in the pancreatic tissue (Griesbacher & Lembeck, 1992; Griesbacher *et al*., 1993; 1995). Kinins also may have some importance for the progression of oedematous to necrotizing forms of the disease (Hoffmann *et al*., 1996; Satake *et al*., 1996). Hence, bradykinin B_2 receptor antagonists are currently being further investigated on their effects in different models of acute pancreatitis. However, aside from blockade of kinin receptors, the inhibition of kinin generation could be an alternative strategy for the prevention of kinin-mediated effects provided that the enzyme(s) responsible for the release of endogenous kinins are determined and specific inhibitors for these enzymes are available. Apart from the 'specific' kininogen-cleaving enzymes, the kallikreins, a number of other enzymes, which are also present in the pancreas, such as trypsin, cathepsin B and others, have the potential for kinin release. Investigations on the mechanisms of kinin release during acute pancreatitis have also been hindered by the fact that enzyme inhibitors capable of inhibiting kallikrein activity also inhibit the other enzymes, frequently with much higher potency.

Recently, two series of low molecular weight inhibitors have been developed which are selective inhibitors for tissue kallikrein and plasma kallikrein, respectively (Evans *et al*.,

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1996a,b). In the present investigation we have sought to determine the role of the two forms of kallikrein using the tissue kallikrein inhibitor, (2*S*,2′*R*)-2-(2′-amino-3′-(4″chlorophenyl)propanoylamino)-*N*-(3-guanidinopropyl)-3-(1-naphthyl) propanoamide (FE999024, previously CH-2856; Figure 1a), and the plasma kallikrein inhibitor, ((2′*S*,2″*R*)-4-(2′-(2″-(carboxymethylamino)-3″-cyclohexyl-propanoylamino)-3′-phenyl-propanoylamino)piperidine-1-carboxamide) (FE999026, previously CH-4215; Figure 1b), in acute interstitial-oedematous pancreatitis induced by exocrine hyperstimulation by the cholecystokinin analogue, caerulein, in rats.

Methods

Surgical procedure

Female Sprague Dawley rats (200–250 g body weight) were obtained from the Institute of Laboratory Animal Science and Genetics (University of Vienna, Austria). The animals were anaesthetized with a combination of pentobarbitone sodium (40 mg kg^{-1}, i.p.) and phenobarbitone sodium (20 mg kg^{-1}, i.p.). In order to prevent the breakdown of kinins by kininase II (identical to angiotensin-converting enzyme) in the pancreatic tissue samples taken for the measurements of immunoreactive kinins (in preparation), captopril (50 μmol kg^{-1}) was injected i.p, in all animals; in order to assure comparable experimental conditions in the entire study, captopril was also administered to the rats in the remaining experiments. The tissue kallikrein inhibitor, FE999024, and/or the plasma kallikrein inhibitor, FE999026, were injected i.p. at doses of 7–60 μmol kg^{-1}. A jugular vein was cannulated for the i.v. injection of Evans blue and the infusion of caerulein. A carotid artery was cannulated for regular measurements of haematocrit. The azo dye, Evans blue (5 mg kg^{-1}), which stains albumin and, to a lesser extent, other plasma proteins (Allen & Orahovats, 1948), was injected i.v. 25 min after the administration of FE999024 or FE999026. Five min later, the i.v. infusion of caerulein (4 nmol kg^{-1} h^{-1} for 2 h) was started; control animals were infused with phosphate-buffered-saline (4 ml kg^{-1} h^{-1}) instead.

All animal experiments followed the Principles of Laboratory Animal Care (NIH) and the Austrian Law on Experiments in Living Animals. Permission for the experiments was granted by the Commission for Animal Experiments of the Austrian Ministry of Science.

Quantification of haematocrit and hypovolaemia

For the measurements of haematocrit small samples (about 40 μl) of arterial blood were drawn from the carotid artery. These measurements were made 10 min before, immediately prior to the start, and 10, 25, 45, 60, 75, 90, 105 and 120 min after the start of the infusion of caerulein. The percentage changes in the circulating blood volume and plasma volume were calculated according to the formulae given in Griesbacher *et al*. (1993).

Quantification of oedema formation and plasma protein extravasation

At the end of the experiment, the animals were perfused *via* the aorta with 40 ml phosphate-buffered saline to which heparin (10 u l^{-1}) had been added. The splenic portion of the pancreas was excised and weighed. After drying in a vacuum centrifuge, the tissue samples were weighed again. The fluid content of the tissue was calculated as the difference between the wet weight and the dry weight of the samples. The water content of the pancreas is given as fluid content per dry weight of the tissues. Each sample was incubated in 8 ml formamide at 55°C for 24 h (Gamse *et al*., 1980). The Evans blue extracted into the supernatant was measured photometrically at a wavelength of 520 nm (Saria & Lundberg, 1983). The Evans blue content of the tissues is given as μg dye per g dry weight of the tissues.

Measurements of enzyme activities in the blood serum and in the pancreatic tissue

For experiments involving the determination of amylase, lipase, and kallikrein-like activity in the pancreatic tissue separate groups of rats were used. At the end of the caerulein infusion the animals were sacrificed by decapitation and subsequent exsanguination. The trunk blood was collected to determine enzyme activities in the blood serum. The pancreas was excised, cut into smaller pieces and placed in 2 ml cold (4°C) 154 mM NaCl solution. The samples were centrifuged immediately at 4°C at 2×10^5 m s^{-2} for 25 min. The supernatant was then stored at −80°C until assayed. Amylase and lipase activities in the samples were determined using standard substrate assays (Roche, Basel, Switzerland) on a Cobas Fara Autoanalyser (Roche). For the measurement of tissue concentrations of glandular (tissue) kallikrein-like activity samples of the pancreatic tissue were excised and immediately placed in liquid nitrogen. After homogenization, the samples were diluted serially up to 1 : 128 in Tris buffer. Activities of tissue kallikrein and plasma kallikrein were determined photometrically using the chromogenic substrates S-2266 (D-Val-Leu-Arg-p-nitroanilide) and S-2302 (D-Pro-Phe-Arg-p-nitroanilide), respectively, as described by Amundsen *et al*. (1979) and Ito & Statland (1981). All measurements were done in duplicate. A purified kallikrein preparation of porcine pancreas was used to control the performance of the system.

Inhibitory activity of kallikrein inhibitors in vitro

Inhibition activity *in vitro* was determined using the fluorogenic substrate *H*-D-Val-Leu-Arg-AFC for human tissue kallikrein, the chromogenic substrates S2302 and S2222 for human plasma kallikrein and human trypsin respectively. The enzyme was incubated at 37°C with three different concentrations of the fluorogenic or chromogenic substrates and various concentrations of the test compound. Residual enzyme activity (initial rate of reaction) was determined by measuring the change in optical absorbence at 405 nm and the inhibitory constant K_i for the test compound was determined from a Dixon plot (Shori *et al*., 1992).

Substances

FE999024 ((2*S*,2*R*)-2-(2′-amino-3′-(4″chlorophenyl)propanoylamino)-*N*-(3-guanidinopropyl)-3-(1-naphthyl)propanoamide; previous name CH-2856) and FE999026 ((2′*S*,2″*R*)-4-(2′(2″-

(carboxymethylamino)-3″-cyclohexyl-propanoylamino)-3′-phenyl-propanoylamino)piperidine-1-carboxamidine) were synthesized by Ferring Research Limited (Southampton, U.K.) and were dissolved in 154 nM NaCl solution at concentrations of 7–60 μmol ml^{-1}. Caerulein (Sigma Chem.Co., St. Louis, MO, U.S.A.) was dissolved in phosphate-buffered saline (composition in mM: NaCl 136.9, KCl 2.7, KH_2PO_4 1.5, Na_2HPO_4 7.7; p*H* 7.4). All salts were of analytical grade and were obtained from Merck (Darmstadt, Germany). Further substances were: pentobarbitone sodium (Nembutal, Sanofi Santé Animale, Libourne, France), phenobarbitone sodium (Apoka, Vienna, Austria), Evans blue (Sigma), formamide (Merck), S-2266 (COA-Chrom Diagnostica, Vienna, Austria), S2222 and S2302 (Quadratech, Epsom, Surrey, U.K.), *H*-D-Val-Leu-Arg-AFC (Bachem Ltd., St. Helens, Lancs., U.K.), heparin (Immuno AG, Vienna, Austria), human tissue kallikrein, human plasma kallikrein and human trypsin (CN Biosciences Ltd, Nottingham, U.K.).

Data analysis

Comparisons between different treatment groups were made using nonparametric analysis of variance and multiple comparisons for independent data (Zar, 1984). Probability values of $P<0.05$ were considered significant. All values presented are arithmetical means with s.e.mean. The dose-dependency of the effects was tested by the Jonckheere test for ordered alternatives (Siegel & Castellan, 1988).

Results

Selectivity of the kallikrein inhibitors in vitro

The selectivity of FE999024 and FE999026 with respect to inhibition of tissue and plasma kallikrein was tested *in vitro* using the tripeptide derivatives *H*-D-Val-Leu-Arg-AFC and S2302 as substrates, respectively (Table 1). Both compounds showed inhibition of one of the enzymes with a K_i value in the low nM range. FE999024 thus proved to be a potent inhibitor of human tissue kallikrein. Human plasma kallikrein was inhibited only at much higher concentrations (K_i1.0 μM), so that the selectivity of FE999024 for tissue vs plasma kallikrein is about 1 : 200. In contrast, FE999026 was a selective inhibitor of plasma kallikrein displaying poor activity for tissue kallikrein (K_i 6.0 μM), so that the selectivity for plasma vs tissue kallikrein is approximately 1 : 2,000. Both inhibitors had a very low inhibitory potency when tested against human trypsin. The K_i values obtained for inhibition of this enzyme were 250–300 fold higher than those for the respective kallikrein (Table 1).

Table 1 K_i values (in nM) of FE999024 and FE999026 against human tissue kallikrein (t-KK), human plasma kallikrein (p-KK) and human trypsin

Compound	K_i (in nM) vs t-KK	K_i (in nM) vs p-KK	*Selectivity for t-KK or p-KK*	K_i vs *trypsin*	*Selectivity for KK* vs *trypsin*
FE999024	5	1000	1 : 200	1285	1 : 257
FE999026	6000	3	1 : 2000	825	1 : 275

K_i values were determined by incubation of the enzyme with the fluorogenic substrate *H*-D-Val-Leu-Arg-AFC (for t-KK), or with the chromogenic substrates S2302 (for p-KK) and S2222 (for trypsin) at 37°C

Effects in experimental pancreatitis

Oedema formation The i.v. infusion of caerulein induced an acute interstitial-oedematous pancreatitis as demonstrated by the increase of the pancreatic water content from basal values of 2.8 ± 0.2 g g^{-1} dry wt ($n=11$) to 11.3 ± 0.9 g g^{-1} dry wt ($n=14$) ($P<0.01$). This effect was inhibited by the tissue kallikrein inhibitor, FE999024, in a dose-dependent manner following the i.p. administration of the compound at doses of 7–60 μmol kg^{-1} (Figure 2a). While the effect of the lowest dose was not significant, both 20 and 60 μmol kg^{-1} of FE999024 resulted in significant ($P<0.05$) attenuations of the increases in water content of the pancreatic tissue in caerulein-treated rats. Caerulein also induced a pronounced extravasation of plasma proteins, quantified as tissue accumulation of the protein marker, Evans blue, which increased from 33 ± 9 μg g^{-1} dry wt ($n=7$) to 373 ± 55 μg g^{-1} dry wt ($n=14$) ($P<0.01$). This effect of caerulein also was reduced in the same, dose-dependent fashion (Figure 2b). The highest dose of FE999024 (60 μmol kg^{-1}, i.p.) completely abolished the plasma protein extravasation ($P<0.05$).

In contrast, the plasma kallikrein inhibitor, FE999026, did not show any inhibitory effect on oedema formation (Figure 2c) or on plasma protein extravasation (Figure 2d). Both kallikrein inhibitors had no significant effect on pancreatic water content or Evans blue extravasation in control animals receiving an i.v. infusion of phosphate-buffered saline instead of caerulein.

Haemoconcentration and hypovolaemia Haematocrit values determined in the arterial blood during caerulein-induced pancreatitis increased from basal values of 0.465 ± 0.003 to peak values of 0.520 ± 0.009 ($n=12$) occurring at 60–90 min of the infusion of caerulein. Calculated peak change in circulating plasma volume thus amounted to $-18\pm3\%$ ($P<0.01$). The tissue kallikrein inhibitor, FE999024, significantly ($P<0.05$) attenuated this response following its i.p. administration at a dose of 20 μmol kg^{-1} (Figure 3a). The effects of the lower dose of 7 μmol kg^{-1} and, surprisingly, also of the higher dose of 60 μmol kg^{-1} remained non-significant. Control animals without induction of pancreatitis exhibited slight increases in apparent circulating plasma volume ($5\pm3\%$; $n=6$) due to the infusion of phosphate-buffered saline (8 ml kg^{-1} within 2 h). Pretreatment of such animals with FE999024 (60 μmol kg^{-1}) resulted in a value of $-1\pm3\%$ ($n=6$) which might account for the lack of significant inhibition of this dose in caerulein-induced pancreatitis.

Caerulein-induced hypovolaemia was left completely unaffected by the plasma kallikrein inhibitor, FE999026 (7–60 μmol kg^{-1}, i.p.). The highest dose of this inhibitor also seemed to induce a slight loss in circulating plasma volume when given on its own. This effect (-4 to -10%), however, occurred only in three out of six rats, whereas the remaining animals exhibited an increase of this parameter ($+3$ to $+8\%$) as seen in phosphate-buffered saline-infused control rats ($3\pm3\%$; $n=6$) where FE999026 was replaced by its vehicle, 154 mM NaCl solution.

Accumulation of digestive enzymes in the pancreatic tissue
Basal values of amylase in the pancreatic tissue amounted to 0.6 ± 0.1 u mg^{-1} dry wt ($n=11$). During caerulein-induced pancreatitis this value was significantly ($P<0.01$) increased to 12.2 ± 1.4 u mg^{-1} dry wt ($n=13$) demonstrating the intrapancreatic activation and accumulation of digestive enzymes

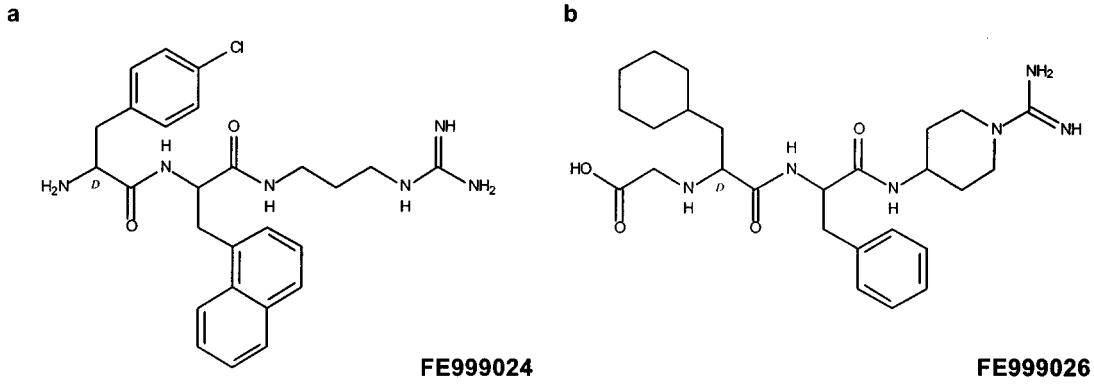

Figure 1 Chemical structure of (a) the tissue kallikrein inhibitor, FE999024 ((2*S*,2′*R*)-2-(2′-amino-3′-(4″chlorophenyl)propanoylamino)-*N*-(3-guanidinopropyl)-3-(1-naphthyl)propanoamide; $C_{26}H_{31}ClN_6O_2$, mol. wt. 495.03), and (b) the plasma kallikrein inhibitor, FE999026 ((2′*S*,2″*R*)-4-(2′-(2″)-carboxymethylamino)-3″-cyclohexyl-propanoylamino)-3′-phenyl-propanoylamino)piperidine-1-carboxamidine; $C_{26}H_{40}N_6O_4$, mol. wt. 500.65).

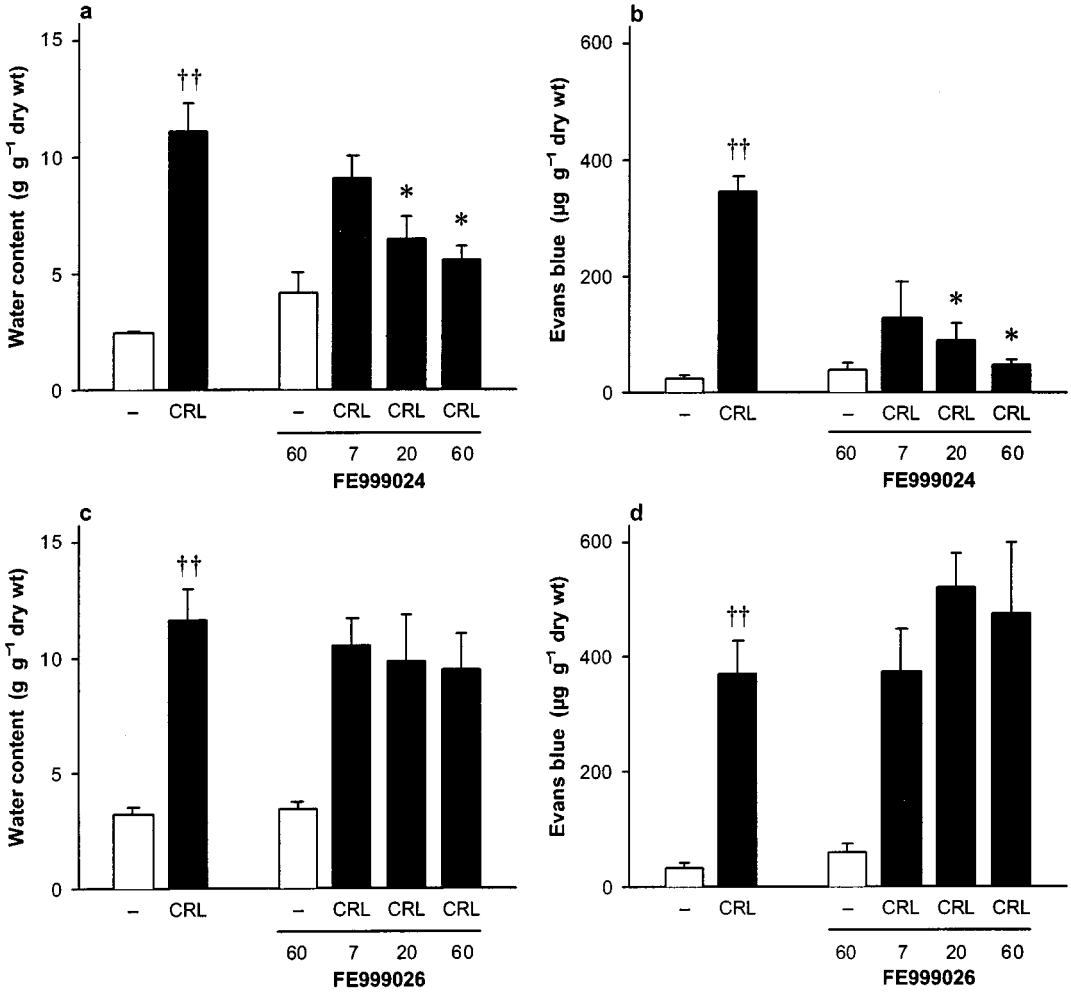

Figure 2 Effect of (a,b) the tissue kallikrein inhibitor, FE999024 (7–60 μmol kg^{-1}), and (c,d) the plasma kallikrein inhibitor, FE999026 (7–60 μmol kg^{-1}), on pancreatic oedema formation (a,c) and plasma protein extravasation (b,d) during acute pancreatitis induced by caeruelin (CRL; 4 nmol kg^{-1} h^{-1} i.v. for 2 h).The inhibitors were given i.p. 30 min prior to the infusion of caerulein or its vehicle, phosphate-buffered saline (4 ml kg^{-1} h^{-1}). Control animals not receiving an inhibitor were injected i.p. with 154 mM NaCl solution (1 ml kig^{-1}). Significance of difference of caerulein *vs* saline infusion. ††$P<0.01$, significance of difference from caerulein without kallikrein inhibitor: *$P<0.05$; means + s.e.mean; $n=5-14$.

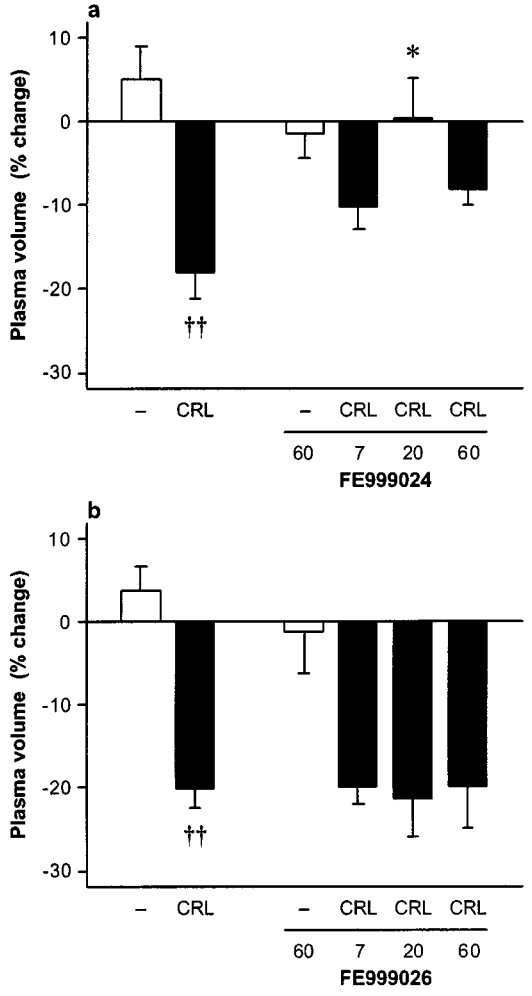

Figure 3 Effect of (a) the tissue kallikrein inhibitor, FE999024, and (b) the plasma kallikrein inhibitor, FE999026, on hypovolaemia during caerulein-induced pancreatitis. The inhibitors (7–60 μmol kg^{-1}, i.p.) or their solvent (154 mM NaCl, 1 ml kg^{-1}), were administered 30 min prior to the start of the infusion of caerulein (CRL; 4 nmol kg^{-1} h^{-1} for 2 h) or its vehicle, phosphate-buffered saline (4 ml kg^{-1} h^{-1}). Peak changes in circulating plasma volume (in per cent) were calculated from repeated measurements of haematocrit in arterial blood. Significance of difference of caerulein *vs* saline infusion: ††$P<0.01$, significance of difference from caerulein without kallikrein inhibitor: *$P<0.05$; means + s.e.mean; $n=5-10$.

during the inflammatory injury. Pretreatment with the tissue kallikrein inhibitor, FE999024 (7–60 μmol kg^{-1}, i.p.), caused a dose-dependent reduction of amylase accumulation in the tissue (Figure 4a); the effect was statistically significant at doses of 20 and 60 μmol kg^{-1}. The plasma kallikrein inhibitor, FE999026, had no effect on this parameter at all doses (Figure 4c). The basal amylase activity in the pancreatic tissue remained unaffected by both compounds.

In the blood serum, amylase activity was increased from basal values of 3.8 ± 0.5 u ml^{-1} ($n=11$) to 13.3 ± 0.9 ($n=13$; $P<0.01$) during caerulein-induced pancreatitis. Following the pretreatment with FE999024 at 60 μmol kg^{-1} i.p., this increase was further augmented to 17.4 ± 1.8 u ml^{-1} ($n=8$; $P<0.05$) (Figure 4b), while treatment with FE999026 remained ineffective at all doses (Figure 4d). Basal amylase activities in the blood serum were not changed by either FE999024 or FE999026.

The i.v. infusion of caerulein also increased the activity of lipase in the pancreatic tissue from 0.6 ± 0.1 u mg^{-1} dry wt ($n=11$) to 6.6 ± 0.8 u mg^{-1} dry wt ($n=13$; $P<0.01$) and in the blood serum from 1.4 ± 0.6 u ml^{-1} ($n=11$) to 5.3 ± 0.6 u ml^{-1} ($n=13$; $P<0.01$). FE999024 (60 μmol kg^{-1}, i.p.) significantly ($P<0.05$) attenuated lipase accumulation in the pancreas (3.8 ± 0.4 u mg^{-1} dry wt, $n=10$; $P<0.05$) and augmented lipase activity in the blood serum (9.0 ± 0.5 u ml^{-1}, $n=10$; $P<0.05$) (not shown in Figure 4) while FE999026 had no effect. The basal activities of lipase in the pancreas or in the blood serum were not changed by any of the two kallikrein inhibitors.

Kallikrein-like activities in the pancreas Tissue kallikrein-like activity in the pancreatic tissue was determined using the synthetic substrate, S-2266. Basal t-KK-like activity in normal pancreas was 73 ± 66 pkat g^{-1} dry wt of the tissue. Following a 2 h infusion of caerulein, t-KK-like activity in the tissue was about 6 fold higher ($P<0.01$) than in the pancreas of control animals without acute pancreatitis (Figure 5a). This increase was completely blocked by the t-KK inhibitor FE999024 as in animals pretreated with this inhibitor no increases in t-KK activity were found in response to caerulein. In contrast, the p-KK inhibitor FE999026 had no effect on the caerulein-induced rises in pancreatic t-KK activity.

The activity of plasma kallikrein (p-KK)-like enzymes in the pancreatic tissue samples was determined using the synthetic substrate, S2302. Basal p-KK-like activity in the tissue was 482 ± 138 pkat g^{-1} dry wt of tissue. During acute pancreatitis, p-KK-like activity was significantly ($P<0.01$) increased about 10 fold (Figure 5b). Pretreatment with either the t-KK inhibitor FE999024 or the p-KK inhibitor FE999026 significantly ($P<0.05$) attenuated p-KK-like activity in the pancreas.

Discussion

Acute pancreatitis is a severe inflammatory disease and is characterized clinically by premature activation of digestive enzymes in the gland, massive oedema formation which may proceed to necrosis, haemoconcentration and hypovolaemia, and severe pain. Although overall mortality rates have decreased due to improved intensive care of the patients, increased incidence rates and improved diagnosis have lead to doubled admission numbers in some university centres (Corfield *et al*., 1985; Wilson & Imrie, 1990) which calls for a better understanding of the pathophysiological mechanisms involved. The majority of patients suffer from the interstitial-oedematous form of the disease (Büchler, 1991) which, despite being self-limiting and eventually leading to full restoration of the organ function, is one of the most painful inflammatory diseases. For the purpose of experimental studies, hyperstimulation of the exocrine function of the pancreas by the cholecystokinin analogue, caerulein (Lampel & Kern, 1977), which leads to derangement of the intracellular membrane processing in acinar cells with subsequent discharge of activated digestive and lysosomal enzymes into the interstitial space (Saluja *et al*., 1987; Willemer *et al*., 1990) is the standard model for this course of the disease as it closely resembles the clinical cases in

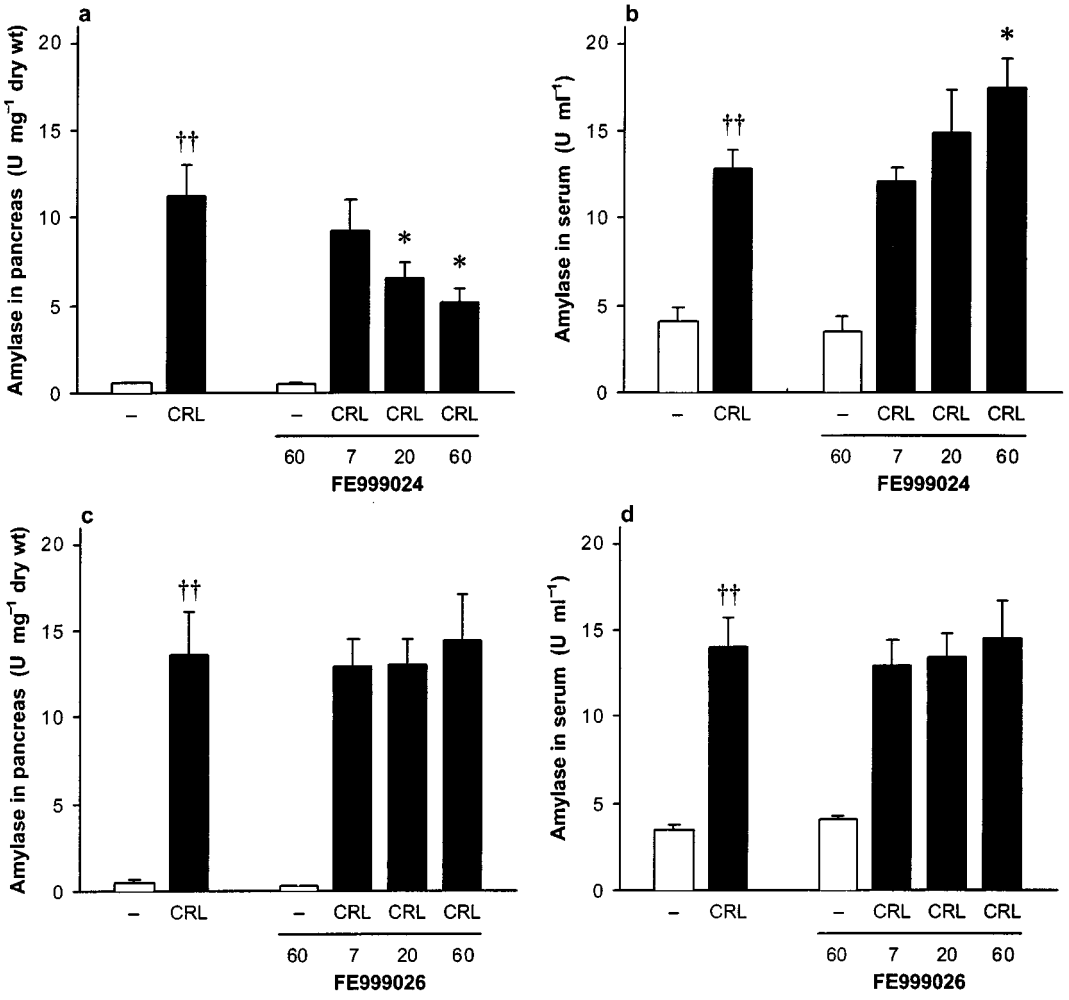

Figure 4 Effect of (a,b) the tissue kallikrein inhibitor, FE999024, and (c,d) the plasma kallikrein inhibitor, FE999026, on amylase activity in the pancreatic tissue (a,c) and in the blood serum (b,d). Acute pancreatitis was induced by i.v. infusion of caerulein (CRL; 4 nmol kg^{-1} h^{-1} for 2 h) while control animals were infused with phosphate-buffered saline (4 ml kg^{-1} h^{-1}). The kallikrein inhibitors (7–60 μmol kg^{-1}) or their vehicle (154 mM NaCl solution, 1 ml kg^{-1}) were injected i.p. 30 min prior to the start of the infusion. Significance of difference of caerulein *vs* saline infusion: ††$P<0.01$, significance of difference from caerulein without kallikrein inhibitor: *$P<0.05$; means + s.e.mean; $n=5-13$.

morphological, ultrastructural, biochemical and functional aspects (Willemer *et al*., 1992).

Investigations using bradykinin B_2 receptor antagonists such as icatibant (Hoe-140) have demonstrated that the increased vascular permeability which is responsible for the oedema formation in the pancreas and for the ensuing hypovolaemia and accumulation of activated enzymes in the pancreatic tissue almost exclusively relies on the intrapancreatic release of endogenous kinins (Griesbacher & Lembeck, 1992; Griesbacher *et al*., 1993; 1995). However, it is not known at present, which enzymes are responsible for the actual generation of kinins. Earlier clinical observations have indicated that both tissue kallikrein and plasma kallikrein may be activated during acute pancreatitis (Uehara *et al*., 1989). In addition to kallikreins, the specific kininogenases, also other enzymes which may be activated during pancreatitis such as trypsin, cathepsins, plasmin and others have the potential for cleavage of kininogens and release of kinins (Prado, 1970). Polyvalent protease inhibitors like aprotinin have originally been introduced in the clinical therapy of pancreatitis specifically with the intention of blocking kinin release by these enzymes (Haberland & McConn, 1979), however, due to their broad spectrum of inhibitory actions they are not suitable for the determination of the pathophysiological role of any one specific enzyme. The recent development of selective inhibitors for tissue and plasma kallikrein (Evans *et al*., 1996a,b) now allows more detailed investigations on the role of kallikreins.

The two compounds used in the present study, FE999024 and FE999026, are selective inhibitors of tissue and plasma kallikrein, respectively. The *in vitro* activity of the compounds (compare Table 1), tested against tissue and plasma kallikrein of human origin, showed that FE999024 has an approximately 200 fold selectivity for the tissue form of kallikrein. FE999026 is even more selective for plasma kallikrein with an approximate 2000 fold selectivity over tissue kallikrein. The residual activity of FE999024 on plasma kallikrein *in vitro* is unlikely to be of relevance for *in vivo* studies unless high doses of the inhibitor are used. In the present investigation, doses of up to 60 μmol kg^{-1} of FE999024 were utilized so that contribution of plasma kallikrein inhibition, despite being unlikely, cannot be ruled out entirely. The use of

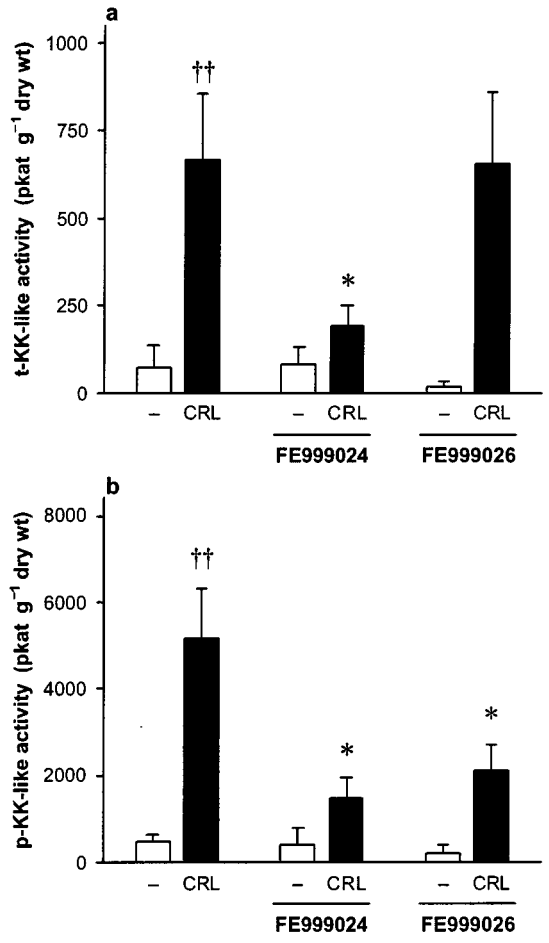

Figure 5 Effect of the tissue kallikrein (t-KK) inhibitor, FE999024, and the plasma kallikrein inhibitor, FE999026, on (a) t-KK-like activity and (b) p-KK-like activity in the pancreatic tissue, determined using the synthetic substrates, S-2266 and S2302, respectively. Acute pancreatitis was induced by i.v. infusion of caerulein (CRL; 4 nmol kg^{-1} h^{-1} for 2 h) while control animals were infused with phosphate-buffered saline (4 ml kg^{-1} h^{-1}). The kallikrein inhibitors (20 μmol kg^{-1}) or their vehicle (154 mM NaCl solution, 1 ml kg^{-1}) were injected i.p. 30 min prior to the start of the infusion. Significance of difference of caerulein *vs* saline infusion: ††$P<0.01$; significance of difference from caerulein without kallikrein inhibitor: *$P<0.05$; means + s.e.mean; $n=4-8$.

FE999026 as a reference compound therefore is necessary to make a definite conclusion as this inhibitor clearly would detect any role of plasma kallikrein. A lack of effect of FE999026 in cases where FE999024 shows inhibition hence can exclude an involvement of the latter form of kallikrein. Likewise, the residual, rather weak activity of both inhibitors on trypsin does not interfere with the interpretation of the *in vivo* results as both agents have a similar inhibitory effect on this enzyme. Inhibition on an observed effect by only one of the compounds and inactivity of the other agent, as observed in the present study (see below), hence excludes trypsin inhibition as the mechanism of action.

The inflammatory oedema of the pancreas resulting from exocrine hyperstimulation by caerulein in the present model was dose-dependently inhibited by the tissue kallikrein inhibitor FE999024 both in terms of extravasation of fluid, determined by the water content of the tissue, and in terms of plasma protein extravasation, measured by Evans blue accumulation in the pancreatic tissue (compare Figure 2). The highest dose of FE999024 used in the present study, i.e. 60 μmol kg^{-1}, caused an almost complete inhibition of oedema formation, which demonstrates that tissue kallikrein plays a central role in the development of the inflammatory increases in vascular permeability in this model of pancreatitis. This compares well with the almost complete inhibition of pancreatic oedema formation by inhibitors of kinin B_2 receptors such as icatibant or FR173657 (Griesbacher & Legat, 2000). The kinin-mediated pancreatic oedema formation has been shown previously to be the cause of a dramatic hypotension due to hypovolaemia with a concomitant haemoconcentration (Griesbacher *et al*., 1993). The hypovolaemia observed during the course of caerulein-induced pancreatitis was somewhat smaller (about 20% reduction in circulating plasma volume) than that observed in our previous investigation (about 48% loss of plasma volume). This effect was again abolished by FE999024 (compare Figure 3).

The effects of FE999024 on amylase activity in the pancreas and in the blood serum (Figure 4) at first seem contradictory as the tissue kallikrein inhibitor strongly reduces the enzyme activity in the pancreatic tissue, but increases them in the blood serum. This, however, is in agreement with the effects of kinin B_2 antagonists (Griesbacher & Legat, 2000). The changes in enzyme activities can be explained by an improved removal from activated enzymes from the tissue into the general circulation when the oedema formation is absent (Griesbacher *et al*., 1995). While a similar effect of the B_2 receptor antagonist in caerulein-induced pancreatitis was reported by Lerch *et al*. (1995), blockade of B_2 receptors seemed to have an attenuating effect on serum amylase levels in taurocholate-induced necrotizing pancreatitis (Bloechle *et al*., 1994) or pancreatitis induced by bilopancreatic duct obstruction (Hirata *et al*., 2002). Therefore, it cannot be ruled out that the role of kinin-mediated effects in enzyme movements from the pancreatic tissue towards the general circulation may be different in different models of acute pancreatitis, which also certainly differ with respect to the grade of severity. In addition, differences with respect to the time of measurements may also be responsible for such discrepancies, especially since we have reported that increased removal of activated digestive enzymes from the pancreatic tissue could only be observed during the acute stage (2 h) of pancreatitis, but not at later time points (Griesbacher *et al*., 1995). It is important to note that neither kallikrein inhibitors nor kinin antagonists interfere with the induction by caerulein of acute pancreatitis but only inhibit generation or action, respectively, of kinins during the inflammatory process. The initial damage to the acinar cells, demonstrated by a typical vacuolization of the cells in histological sections of the pancreas, is not affected by the kallikrein inhibitors or by kinin antagonists (data not shown).

As in all these experiments FE999024 actively inhibited kinin-mediated phenomena while FE999026 remained completely inactive even at a dose of 60 μmol kg^{-1}. The active doses of FE999024 compare well with those found for this compound (then called CH-2856) previously in a model of allergic inflammation in guinea-pigs (2.5–20 μmol kg^{-1}; Evans *et al*., 1996a). The effect of FE999024 on tissue

kallikrein activity could also be confirmed by the determination of tissue kallikrein-like activity in the pancreatic tissue *in vivo* (compare Figure 5a).

Effects of the plasma kallikrein inhibitor in an *in vivo* model in the rat have not been published so far, so that a direct comparison of the doses used in the present investigation is not possible. However, the K_i value of FE999026 *vs* plasma kallikrein *in vitro* is almost identical to the K_i value of FE999024 *vs* tissue kallikrein (see Table 1) so that FE999026 should have caused an inhibition if plasma kallikrein were involved. Indeed, p-KK-like activity appears in the pancreatic tissue during acute pancreatitis (Figure 5b). FE999026 strongly attenuated the elevations in p-KK-like activity, confirming that the dose if FE999026 (20 μmol kg^{-1}) is an effective dose. Since this dose of FE999026, however, had no effect on the vascular effects mediated by kinins (oedema formation, plasma protein extravasation, haemoconcentration etc.), it must be concluded that p-KK-like enzymes are activated during acute pancreatitis, but do not contribute to kinin release. The reduction in p-KK-like activity in the pancreas observed in rats pretreated with the t-KK inhibitor FE999024 can be explained by the prevention of plasma protein extravasation (compare Figure 2b) and consequently prevention of influx of p-KK from the plasma into the tissue.

Taken together the results of the present investigation show that the vascular, kinin-mediated symptoms during acute caerulein-induced oedematous-interstitial pancreatitis in the rat are due to the action of tissue kallikrein but not plasma kallikrein. Selective inhibition of tissue kallikrein by FE999024 proved to be as effective as inhibition of kinin B_2 receptors and thus offers a new pharmacological approach in experimental research in models of inflammation and also could have a therapeutical potential in clinical conditions of such diseases.

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References

ALLEN, T.H. & ORAHOVATS, P.D. (1948). Spectrophotometric measurements of traces of the dye T-1824 by extraction with cellophane from blood serum and urine of normal dogs. *Am. J. Physiol.,* **154,** 27–38.

AMUNDSEN, E., PUTTER, J., FRIBERGER, P., KNOS, M., LARSBRATEN, M. & CLAESON, G. (1979). Methods for the determination of glandular kallikrein by means of a chromogenic tripeptide substrate. *Adv. Exp. Med. Biol.,* **120A:** 83–95.

BLOECHLE, C., KÜHN, R., KNOEFEL, W.T., YEKEBAS, E., KUSTERER, K. & IZBICKI, J.R. (1994). Bradykinin-antagonist Hoe 140 prevents microcirculatory statis and tissue necrosis in taurocholate induced pancreatitis of the rat. *Pancreas,* **9,** 783.

BÜCHLER, M. (1991). Objectification of the severity of acute pancreatitis. *Hepatogastroenterology,* **38,** 101–108.

CORFIELD, A.P., COOPER, M.J. & WILLIAMSON, R.C.N. (1985). Acute pancreatitis: a lethal disease of increasing incidence. *Gut,* **26,** 724–729.

EVANS, D.M., JONES, D.M., PITT, G.R., ASHWORTH, D., DE CLERCK, F., VERHEYEN, F. & SZELKE, M. (1996a). Synthetic inhibitors of human tissue kallikrein. *Immunopharmacology,* **32,** 117–118.

EVANS, D.M., JONES D.M., PITT, G.R., SUEIRAS-DIAZ, J., HORTON, J., ASHWORTH, D., OLSSON, H. & SZELKE, M. (1999b). Selective inhibitors of plasma kallikrein. *Immunopharmacology,* **32,** 115–116.

FORRELL, M. (1955). Zur Frage des Entstehungsmechanismus des Kreislaufkollapses bein der akuten Pankreasnekrose. *Gastroenterologia,* **84,** 225–251.

GAMSE, R., HOLZER, P. & LEMBECK, F. (1980). Decrease of substance P in primary afferent neurones and impairment of neurogenic plasma extravasation by capsaicin. *Br. J. Pharmacol.,* **68,** 207–213.

GRIESBACHER, T., KOLBITSCH, C., TIRAN, B. & LEMBECK, F. (1995). Effects of the bradykinin antagonist, icatibant (Hoe 140), on pancreas and liver functions during and after caerulein-induced pancreatitis in rats. *Naunyn-Schmiedeb. Arch. Pharmacol.,* **352,** 557–564.

GRIESBACHER, T. & LEGAT, F.J. (2000). Effects of the non-peptide B_2 receptor antagonist FR173657 in models of visceral and cutaneous inflammation. *Inflamm. Res.,* **49,** 535–540.

GRIESBACHER, T. & LEMBECK, F. (1992). Effects of the bradykinin antagonist, HOE 140, in experimental acute pancreatitis. *Br. J. Pharmacol.,* **107,** 356–360.

GRIESBACHER, T., TIRAN, B. & LEMBECK, F. (1993). Pathological events in experimental acute pancreatitis prevented by the bradykinin antagonist, Hoe 140. *Br. J. Pharmacol.,* **108,** 405–411.

HABERLAND, G. & MCCONN, R. (1979). A rationale for the therapeutic action of aprotinin. *Fed. Proc.,* **38,** 2760–2767.

HIRATA, M., HAYASHI, I., YOSHIMURA, K., ISHII, K.-I., SOMA, K., OHWADA, T., KAKITA, A. & MAJIMA, M. (2002). Blockade of bradykinin B_2 receptor suppresses acute pancreatitis induced by obstruction of the biliopancreatic duct in rats. *Br. J. Pharmacol.,* **135,** 29–36.

HOFFMANN, T.F., WALDNER, H. & MESSMER, K. (1996). The bradykinin antagonist CP-0597 can limit the progression of postischemic pancreatitis. *Immunopharmacology,* **33,** 243–246.

ITO, R. & STATLAND, B.E. (1981). Centrifugal analysis for plasma kallikrein activity, with use of the chromogenic substrate S-2302. *Clin. Chem.,* **27,** 586–593.

LAMPEL, M. & KERN, H.F. (1977). Acute interstitial pancreatitis in the rat induced by excessive doses of a pancreatic secretagogue. *Virchows Arch. (Pathol. Anat.),* **373,** 97–117.

LERCH, M.M., WEIDENBACH, H., GRESS, T. & ADLER, G. (1995). Effect of kinin inhibition in experimental acute pancreatitis. *Am. J. Physiol.,* **269,** G490–G499.

PRADO, J.L. (1970). Proteolytic enzymes as kininogenases. *Handb. Exp. Pharmacol.,* **25,** 156–192.

SALUJA, A.K., HASHIMOTO, S., SALUJA, M., POWERS, R.E., MELDOLESI, J. & STEER, M.L. (1987). Subcellular redistribution of lysosomal enzymes during caerulein-induced pancreatitis. *Am. J. Physiol.,* **253,** G508–G516.

SARIA, A. & LUNDBERG, J.M. (1983). Evans blue fluorescence: quantitative and morphological evaluation of vascular permeability in animal tissues. *J. Neurosci. Meth.,* **8,** 41–49.

SATAKE, K., HA, H.H. & HIURA, A. (1996). Effects of bradykinin receptor antagonist on the release of beta-endorphin and bradykinin and on hemodynamic changes in a canine model of experimental acute pancreatitis. *Pancreas,* **12,** 92–97.

SHORI, D.K., PROCTER, G.B., CHAO, J., CHAN, K.M. & GARRETT, J.R. (1992). New specific assays for tonin and tissue kallikrein activities in rat submandibular glands. Assays reveal differences in the effects of sympathetic and parasympathetic stimulation on proteinases in saliva. *Biochem. Pharmacol.,* **43,** 1209–1217.

SIEGEL, S. & CASTELLAN JR, N.J. (1988). Nonparametric Statistics for the Behavioral Sciences 2nd ed. New York: McGraw-Hill.

UEHARA, S., HONJYO, K., FURUKAWA, S., HIRAYAMA, A. & SAKAMOTO, W. (1989). Role of the kallikrein-kinin system in human pancreatitis. *Adv. Exp. Med. Biol.,* **247B,** 157–162.

WERLE, E., TAUBER, K, HARTENBACH, W. & FORRELL, M.M. (1958). Zur Frage der Therapie der Pankreatitis. *Münch. Med. Wochenschr.,* **100,** 1265–1267.

WILLEMER, S., BIALEK, L. & ADLER, G. (1990). Localization of lysosomal and digestive enzymes in cytoplasmic vacuoles in caerulein-pancreatitis. *Histochemistry,* **94,** 161 – 170.

WILLEMER, S., ELSÄSSER, H.-P. & ADLER, G. (1992). Hormone-induced pancreatitis. *Eur. Surg. Res.,* **24 (Suppl. 1):** S29 – S39.

WILSON, C. & IMRIE, C.W. (1990). Changing patterns of incidence and mortality from acute pancreatitis in Scotland, 1961 – 1985. *Br. J. Surg.,* **77,** 731 – 734.

ZAR, J.H. (1984). *Biostatistical analysis*. 2nd ed. Englewood Cliffs, N.J.: Prentice-Hall.

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