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Degradation of bradykinin by bovine tracheal epithelium and isolated epithelial cells

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1 The degradation of bradykinin (BK) labelled with tritiated proline at positions 2 and 3 ([^{3}H]-BK) was determined on the luminal surface of bovine tracheal epithelium, in supernatants obtained from incubations of the luminal tracheal surface, and in suspensions of isolated tracheal epithelial cells. Peptidase inhibitors and identification of peptide fragments were used for characterization of the metabolic pathways.

2 On the luminal surface of intact bovine trachea, [^{3}H]-BK was degraded with a half life of 12.8 min. [1-7]-BK and [1-5]-BK were the major direct metabolites which were further degraded via [1-3]-BK and [2-3]-BK to proline. Metabolism of [^{3}H]-BK was unaltered in the presence of ramiprilat (250 nM) or phosphoramidon (10 μM). Phenanthroline diminished the formation of [1-7]- and [1-5]-BK and abolished the generation of proline.

3 Supernatants obtained from incubations of tracheal epithelium contained kininase activities which steadily increased when tracheae were incubated for longer than 30 min. After 60 min contact with epithelium, the incubation medium contained higher kininase activities than the epithelium itself. The spectrum of kinin metabolites generated by kininases in the supernatant was comparable to that formed by intact epithelium.

4 In suspensions of isolated epithelial cells, [^{3}H]-BK was degraded with a half life of 70 min. The metabolites [1-3]- and [2-3]-BK were formed in parallel to [1-7]- and [1-5]-BK; however, proline was not generated. Degradation of [^{3}H]-BK was not influenced by ramiprilat, but was inhibited by 85% in the presence of phosphoramidon. Phosporamidon markedly inhibited the generation of [1-7]- and [1-5]-BK and nearly abolished the formation of [1-3]- and [2-3]-BK.

5 In conclusion, angiotensin I-converting enzyme and neutral endopeptidase 24.11 are not significantly involved in [^{3}H]-BK degradation on the luminal side of intact tracheal epithelium. The spectrum of metabolites found may in fact reflect the combined activities of metalloendopeptidase 24.15 and post-proline cleaving enzyme. Enzymes showing similar kininase activities are also released from the epithelium. Isolated epithelial cells contain low activities of these kininases, but a high activity of neutral endopeptidases, which may reflect an exclusively basolateral localization of the latter.

Keywords: Bradykinin; kininases; kinin metabolism; angiotensin I-converting enzyme; neutral endopeptidase; ramiprilat; phosphoramidon; bovine; trachea; bronchial epithelium

Introduction

Kinins have for long been considered to be involved as mediators in inflammatory and obstructive pulmonary diseases (Baumgarten *et al*., 1992; Polosa, 1993). This is due to the pronounced exudative properties of these peptides (Lötvall *et al*., 1991) in conjunction with the bronchoconstrictive effect which can be shown *in vitro* and after systemic administration or inhalation of kinins in various animal models (Collier, 1970; Dusser *et al*., 1988).

Under pathophysiological conditions kinins are generated at the intraluminal side of the airway epithelium since kinins, as well as tissue kallikrein, have been detected in bronchoalveolar lavage fluids from asthmatics (Christiansen *et al*., 1987; Baumgarten *et al*., 1992). At this location kinins can act on epithelial surfaces and glandular cells to stimulate Cl^- secretion (Leikauf *et al*., 1985), ciliary activity (Tamaoki *et al*., 1989) and the release of prostanoids which have been shown to possess both bronchodilator (Bramley *et al*., 1990) and bronchoconstrictor (Molimard *et al*., 1995) properties. Counterregulation of bronchoconstriction by the epithelium may involve an, as yet, unidentified mechanism (Barnes *et al*., 1985). Evidence for the physiological significance of this property arises from the finding that inhaled bradykinin has a strong constrictor effect only in asthmatics (Fuller *et al*., 1987), which may be due to the disturbed integrity of the bronchial epithelium found in those patients (Beasly *et al*., 1989). In the bronchial wall kinins can exert additional activities, the most important of which are contraction of smooth muscle cells and induction of microvascular leakage either of which can lead to airway obstruction. These effects can be produced not only by direct actions of kinins on smooth muscle cells and vascular endothelium, but also by indirect mechanisms involving vagal reflexes and/or release of prostanoids, leukotrienes and tachykinins (Saria *et al*., 1988; Ichinose *et al*., 1990; Molimard *et al*., 1995). In addition, afferent C-fibres are stimulated by bradykinin (Inoue *et al*., 1992) and thus kinins can maintain neurogenic mechanisms of inflammation and may be involved in central nociception.

As with other locally acting tissue hormones, the activity of kinins in particular is determined by factors leading to their degradation. Virtually all types of kininases have been identified by biochemical means in lung or bronchial tissues. These include carboxypeptidase M (EC 3.4.24.-) (Nagae *et al*., 1993), angiotensin I-converting enzyme (ACE, EC 3.4.15.1) which is particularly prevalent in the lung (Ryan *et al*., 1976), neutral endopeptidase (NEP, EC 3.4.24.11) (Zolfaghari *et al*., 1989; Nadel & Borson, 1991), post-proline cleaving enzyme (PPCE, EC 3.4.21.26) (Zolfaghari *et al*., 1986), metallo-endopeptidase (MEP, EC 3.4.24.15) (Chu & Orlowski, 1985) and aminopeptidase P (EC 3.4.11.9) (Sidorowicz *et al*., 1984). The studies mentioned have either been performed on tissue homogenates

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or have investigated the vascular degradation of kinins so that no location-specific information about kininase activities could be obtained. It may therefore be the case that some of the described kininases are confined either to the cytosol (like PPCE) or to the vascular system (like ACE and aminopeptidase P) so that they do not contribute towards intraluminal or epithelial mechanisms of kinin degradation. Functional studies on the potentiation of bronchoconstriction and increase of vascular permeability induced by bradykinin also warrant such considerations on site-specificity of kininases, since they have mostly demonstrated the importance of ACE and NEP but not of other enzymes (Dusser *et al*., 1988; Lötvall *et al*., 1991).

Further interest in ACE arose from clinical observations on inflammation-like side effects of ACE inhibitors, the most frequent of which is a dry cough (Sesoko & Kaneko, 1985). The occurrence of this symptom has been linked hypothetically to an accumulation of bradykinin (BK, the most common physiological kinin in man), brought about by inhibition of the extensive ACE activity which is present in the lung. Some evidence supporting this view was derived from studies which demonstrated that ACE inhibitors can potentiate the bronchial effects of exogenous kinins *in vitro*. However, this enzyme has been only shown to be present on the vascular endothelium from immuno-histological studies (Ryan *et al*., 1976). This indicates that functional studies on tracheae or bronchi where BK was applied at the abluminal side (Dusser *et al*., 1988) or in very high doses at the luminal side (Lötvall *et al*., 1991) must not have primarily assessed kinin degradation pathways at or in the epithelium. This may be relevant for the interpretation of the findings from these studies, because it should not be assumed that the kinins generated in the bronchial lumen or wall will be cleared by vascular enzymes.

A further pathophysiological aspect of bronchial inflammation concerns the capability of the airway epithelium to form not only a metabolic, but also a physical barrier against intrabronchial mediators. If an intact epithelium is able to prevent access of luminal kinins to the bronchial wall, then the integrity and perhaps specific regulatory mechanisms of the epithelium might significantly affect the biological actions of these inflammatory mediators.

In view of the significance of airway epithelium in modulating the biological activities of kinins, we set out to investigate the metabolism of bradykinin by intact tracheal epithelium and focussed in particular on its degradation by the kininase II-type enzymes ACE and NEP. The breakdown of BK was assessed after applying tritiated BK at very low concentrations either to the luminal side of bovine intact trachea, to supernatants obtained thereof, or to freshly isolated tracheal epithelial cells. Degradation pathways were characterized by use of specific peptidase inhibitors and by identification of the BK metabolites generated.

Preparation of bovine trachea

Bovine tracheae were freshly obtained from a local slaughterhouse. The organs were kept at 4°C in sterile transportation medium (DMEM/F12 without $NaHCO_3$ supplemented with 10 mM N-[2-hydroxyethyl]piperazine-N′-2-ethanesulphonic acid, 100000 u l^{-1} penicillin, 0.1 g l^{-1} streptomycin and 0.05 g l^{-1} gentamycin, pH 7.4).

Incubation of intact epithelium

Tracheae were cut lengthwise at the median of the cartilage clasps. After thorough washing with phosphate-buffered saline, pieces of about 10 cm length were mounted on a frame, the epithelial surface facing upside. Incubation areas of 2 cm^2 each were subdivided by pressing the upper rim of a 24-well cell culture dish (Nunc, Wiesbaden, F.R.G.) onto the epithelial surface. The bottoms of the cell culture dishes had been removed in order to permit the exchange of incubation medium (DMEM/F12 without $NaHCO_3$, supplemented with 10 mM N-[2-hydroxyethyl]piperazine-N′-2-ethanesulphonic acid and 0.2% bovine serum albumin, pH 7.4). Tracheae were suspended in albumin-free incubation medium and warmed to 37°C in a waterbath. The incubation areas were washed three times with incubation medium and were then pretreated for 15 min with specific enzyme inhibitors as indicated. Incubations were started by exchanging the supernatants for 1 ml of incubation medium containing 0.43 nM [^{3}H]-BK and the respective inhibitor. At the beginning and after 5, 10, 30, 60, 120 and 180 min, aliquots (70 μl) were sampled, inactivated by addition of 7 μl trifluoroacetic acid (1%) and stored at −80°C until analysed.

For determination of peptidase activities released into the supernatants, intact tracheal epithelium was incubated as described, but [^{3}H]-BK was omitted in the incubation medium. Samples of the supernatants were taken at the timepoints indicated, supplemented with 0.43 nM [^{3}H]-BK immediately, and incubated further for 6 to 60 min at 37°C.

Incubation of isolated epithelial cells

Epithelial cells were isolated according to a method modified from Groelke *et al*. (1985). Briefly, tracheae were washed thoroughly with phosphate buffered saline, closed at both ends with tubing clamps and filled with enzyme solution (2.5 g l^{-1} pronase (Sigma, Deisenhofen, F.R.G.) in transportation medium). After incubation for 18 h at 4°C, the enzyme solution was collected and the luminal surface of each trachea was flushed with 50 ml Ham's F12 medium containing 10% foetal calf serum. Both cell suspensions were combined and centrifuged ($50 \times g$, 10 min, 4°C). The cell pellet was washed three times with transportation medium ($50 \times g$, 10 min, 4°C) and finally resuspended in incubation medium.

For degradation experiments, freshly isolated epithelial cells suspended in 1 ml incubation medium were transferred to multiwell dishes (24-well, Nunc, Wiesbaden, F.R.G.) and pretreated for 15 min with peptidase inhibitors. Incubations were started by addition of [^{3}H]-BK at a final concentration of 0.43 nM and maintained with continuous agitation at 37°C. Aliquots of the medium were sampled at incubation times up to 180 min as described above.

In order to test the viability, identity and sterility of epithelial cell preparations, cell cultures were established in 24-well dishes coated with collagen IV in Ham's F12 culture medium supplemented with 10 mg l^{-1} transferrin, 0.2 mg l^{-1} hydrocortisone, 5 mg l^{-1} insulin, 30 μg l^{-1} ethanolamine, 70 μg l^{-1} phosphoethanolamine, 5 μg l^{-1} epidermal growth factor, 100 000 u l^{-1} penicillin and 100 mg l^{-1} streptomycine. Cell cultures were maintained at 37°C and 5% CO_2. Epithelial cells were identified by double-layer immunofluorescence with mouse anticytokeratin 8.13 as primary and a FITC-labelled goat antimouse as secondary antibody.

Peptide analysis

Peptides were separated on an ET 200 Nucleosil 5 C_{18} column (Macherey-Nagel, Düren, F.R.G.) at 40°C and 0.8 ml min^{-1} with 0.1% trifluoroacetic acid containing acetonitrile/methanol (60:1), at concentrations linear increasing from 1.2% to 25% during 60 min, as eluent. Figure 1 exemplifies a high performance liquid chromatograph (h.p.l.c.) tracing of all investigated peptides (BK, [1-8]-BK, [2-9]-BK, [1-7]-BK, [1-6]-BK, [1-5]-BK, [1-4]-BK, [1-3]-BK, [2-3]-BK, [1-2]-BK and proline), applied as unlabelled standards and detected by absorption at 210 nm. For determination of labelled kinin fragments by radioactivity, eluent was collected in 1.6 ml fractions at the retention times of the unlabelled analogues. Each fraction was mixed with 10 ml scintillation cocktail (Hydroluma, Baker, Deventer, The Netherlands) and analyzed in a β-counter. As internal standard bradykinin (BK) was added to the samples reaching a final concentration of 8 μM. The separation of all kinin fragments except [1-4]-BK and [1-3]-BK was sufficient to collect each peptide selectively into a 1.6 ml

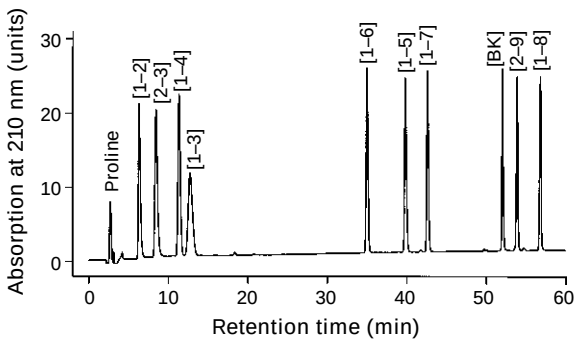

Figure 1 H.p.l.c. separation of bradykinin and kinin metabolites. Standard peptides (5–20 μg) of bradykinin (BK) and of various kinin fragments (indicated by position within the bradykinin sequence) were chromatographed under the conditions described in Methods and detected at 210 nm. The trace demonstrates sufficient resolution to allow discrimination of the single fragments by fractionation. The separation of [1-3]-bradykinin and [1-4]-bradykinin is referred to in Methods.

fraction. Although the peptides [1-4]-BK and [1-3]-BK were resolved to baseline, their separation did not permit selective analysis of both peptides by fractionation. However, the observed absence of radioactivity in the fraction of [1-4]-BK should suffice to exclude the presence of this peptide even on the basis of the h.p.l.c. resolution achieved. At the substrate concentration given, any kinin fragment could be detected which at least amounted to 0.5% of total radioactivity.

Substances

Medium DMEM/F12 (1:1) was obtained as $NaHCO_3$-free salt mixture from Gibco (Eggenstein, F.R.G.). Ham's F12 medium, all antibiotics, cell culture supplements and the mouse anti-cytokeratin 8.13 antibody were bought from Sigma (Deisenhofen, F.R.G.). FITC-labelled goat anti-mouse antibody was obtained from Dako (Hamburg, F.R.G.). Peptide standards BK, [1-8]-BK, [2-9]-BK, [1-7]-BK, [1-6]-BK, [1-5]-BK, [1-3]-BK, [2-3]-BK, [1-2]-BK and proline were bought from either Sigma (Deisenhofen, F.R.G.) or Bachem (Bubendorf, Switzerland). [1-4]-BK was first prepared from [1-5]-BK by incubation with carboxypeptidase A (Sigma, Deisenhofen, F.R.G.), and then purified chromatographically and identified by amino acid analysis. [^{3}H-Pro^{2,3}]-bradykinin ([^{3}H]-BK) was obtained from Du Pont (Bad Homburg, F.R.G.) at a specific activity of 2.22 TBq mmol^{-1}. It was used in the incubation media at an activity of 40000 c.p.m. ml^{-1}, corresponding to a concentration of 0.43 nM. The following peptidase inhibitors were applied: the ACE inhibitors ramiprilat and zofenoprilat, which were generous gift from Hoechst (Frankfurt, F.R.G.) and Squibb, (Regensburg, F.R.G.), respectively; phosphoramidon (10 μM, Sigma, Deisenhofen, F.R.G.), which inhibits the enzyme neutral endopeptidase 24.11, and 1,10-phenanthroline (1 mM, Sigma, Deisenhofen, F.R.G.), which is a nonspecific inhibitor of various metalloproteases.

Quantification and statistics

Suspension cell numbers were determined in a Neubauer counting chamber. Cell density of intact epithelium was determined by morphometry of thin slices cut tangentially to the epithelial layer. For preparation of histological slices, epithelial specimens of formaldehyde-fixated tracheae were dehydrated in a graded series of ethanol and in xylol, embedded in paraffin and cut into 4 μm thick slices. After rehydration, these slices were stained with hematoxylin-eosin using standard procedures. Protein contents of cell suspensions and cultures were measured according to Lowry *et al.* (1951). Peptide concentrations in the incubation solutions were quantified according to the ^{3}H-activities measured in the specific h.p.l.c. fractions. These values were expressed as percentages of the total radioactivity of all kinin metabolites detected in the substrate solution. [^{3}H]-BK contributed 94.3 $\pm$ 0.3% to the total radioactivity of the substrate solution, revealing only minor impurities of the substrate. For analysis of degradation kinetics, the complete time course of [^{3}H]-BK degradation was fitted to a function of monoexponential decline ($BK(t) = BK_o * e^{-\beta * t}$). The velocity of [^{3}H]-BK degradation is always given as the rate constant β or the derived half-life. In experiments with suspensions of freshly isolated epithelial cells, the kinetics of [^{3}H]-BK degradation were not adequately described by a monoexponential decline. In these experiments an initial fast phase of [^{3}H]-BK decline occurred which was paralleled by a reduction of total radioactivity in the samples amounting to 17% of the substrate radioactivity after 10 min incubation. This rapid initial loss of [^{3}H]-BK was attributed to adsorption of peptides to the plastic well surface, since this effect was observed at about the same magnitude (13.4% reduction of total radioactivity after 5 min) in control incubations performed in the absence of cells. The kinetics of [^{3}H]-BK degradation in cell suspensions was therefore fitted to a biexponential equation ($BK(t) = A * e^{-\alpha * t} + B * e^{-\beta * t}$), where the magnitude of the early fast phase (A) was always set to compensate for the loss of total ^{3}H-activity (17% of ^{3}H-activity in the substrate solution). Only the rate constant of the slow phase of [^{3}H]-BK decline (β) was attributed to kinin degradation. All data are given as means $\pm$ s.e.mean from 4 to 7 independent experiments. Some peptidase inhibitors (ramiprilat and zofenoprilat in high concentrations, 1,10-phenanthroline) were only tested twice because of their obvious lack of effect or specificity. The effects of ramiprilat and phosphroamidon were evaluated by analysis of variance for repeated measurements by use of Dunnet's post-hoc test. Differences were considered to be statistically significant at an error level of $P < 0.05$.

Results

Degradation of [^{3}H]-BK by intact tracheal epithelium

On the luminal surface of intact tracheal epithelium, [^{3}H]-BK was degraded with a rate constant of 0.081 $\pm$ 0.018 min^{-1} corresponding to a half-life of 12.8 $\pm$ 3.0 min (Figure 2a). The fragments [1-7]-BK and [1-5]-BK were the predominant degradation products in the early phase of the incubations reaching maximum levels after 30 and 60 min, respectively (Figure 2a). These peptides were generated in parallel and no precursors or intermediates such as [1-8]-BK or [1-6]-BK could be detected. The generation of the fragments [1-7]-BK and [1-5]-BK by intact epithelium suggested the presence of kininase II enzymes, ACE and neutral endopeptidase 24.11 (NEP). We therefore tested the influence of ramiprilat and phosphoramidon as inhibitors of ACE and NEP, respectively. Application of ramiprilat (0.25 μM) during 15 min of pretreatment and throughout the incubations did not alter the rate of [^{3}H]-BK breakdown (rate constant 0.072 $\pm$ 0.020 min^{-1}, half-life 15.6 $\pm$ 3.4 min) (Figure 2b). Ramiprilat appeared to modify the proportions at which [1-5]-BK and [1-7]-BK were generated (Figure 2b). However, the concentrations of both fragments did not significantly differ from those of the control experiments. Phosphoramidon did not alter either the rate of [^{3}H]-BK breakdown (rate constant 0.077 $\pm$ 0.03 min^{-1}, half-life 14.7 $\pm$ 2.6 min) or the qualitative spectrum of kinin metabolites (Figure 2c).

In order to confirm the lack of effect of ACE inhibitors and to exclude atypical substance specificities or dose requirements, we tested ramiprilat and the SH-bearing ACE inhibitor zofenoprilat in concentrations of 2.5 and 25 μM. While both inhibitors were ineffective at 2.5 μM, zofenoprilat and ramiprilat (25 μM) inhibited [^{3}H]-BK breakdown by 32 and 6%, respec-

tively. An effective inhibition of [^{3}H]-BK breakdown was only achieved with 1,10-phenanthroline (1 mM), a non-selective inhibitor of metalloproteases which reduced the activity of tracheal kininases by 50%. These experiments were only repeated twice because they only served to confirm the ineffectiveness of ACE inhibitors even in high concentrations. However, the identity of the kininases could not be determined under such conditions.

In a second set of experiments, we tried to identify further degradation steps and possible end products of kinin metabolism. As metabolites bearing at least one tritiated proline the fragments [1-3]-BK and [2-3]-BK and proline itself could be identified (Figure 3a). Of these, only [1-3]-BK can be produced directly from [^{3}H]-BK, [1-5]-BK or [1-7]-BK. Formation of other precursors of [1-3]-BK and [2-3]-BK, like [1-4]-BK or [2-9]-BK was not observed. For this reason, proline must have been derived primarily from [2-3]-BK (Pro-Pro) and the sum of [1-3]-BK, [2-3]-BK and proline consequently should represent the total formation of [1-3]-BK.

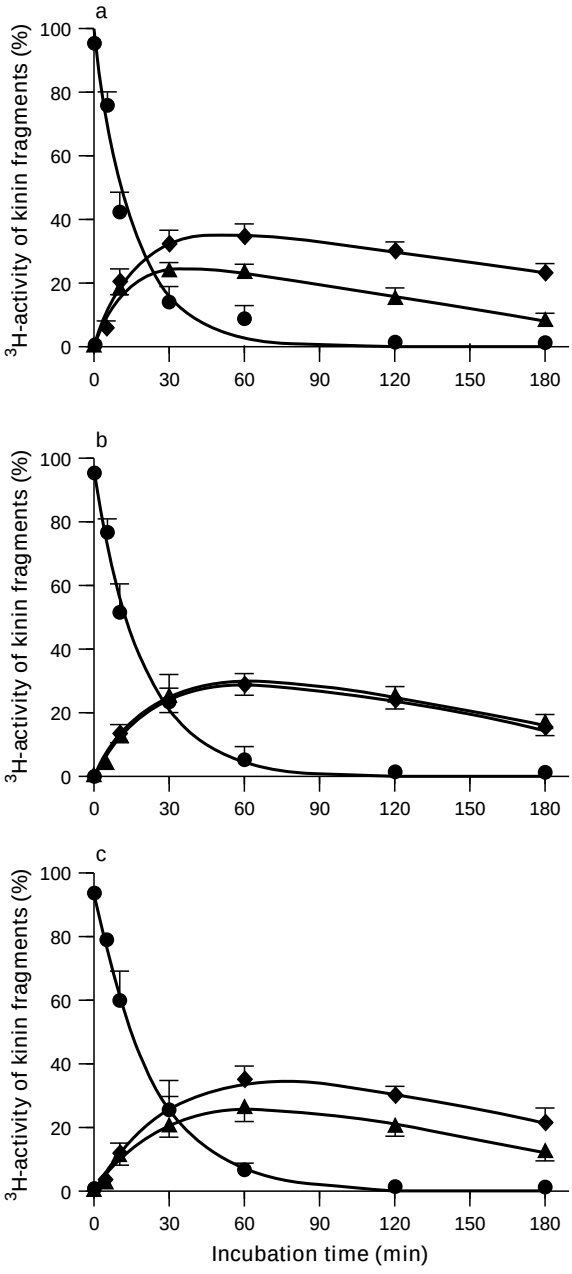

Figure 2 Kinetics of [^{3}H]-bradykinin ([^{3}H]-BK) breakdown by bovine intact tracheal epithelium. Time courses of the relative amounts of [^{3}H]-BK (●) and its degradation fragments [1-7]-BK (▲) and [1-5]-BK (◆) are depicted. The experiments were either performed (a) without peptidase inhibitors or after 15 min preincubation with and in the presence of (b) ramiprilat (0.25 μM) or (c) phosphoramidon (10 μM). Rate constants of [^{3}H]-BK degradation were not altered significantly by these inhibitors ($0.081 \pm 0.018\,min^{-1}$, $0.072 \pm 0.02\,min^{-1}$ and $0.077 \pm 0.03\,min^{-1}$ in control, ramiprilat and phosphoramidon groups, respectively). Each point represents the mean of 7 determinations; vertical lines show s.e.mean.

Taking [1-3]-BK, [2-3]-BK and proline together, the time-course of their total amount was clearly biphasic (Figure 3a). [1-3]-BK and its metabolites were generated at the highest rate between 60 and 120 min of incubation. Transformation of [1-5]-BK or [1-7]-BK into [1-3]-BK could be shown by continued increases in the levels of [1-3]-BK and its metabolites at incubation times longer than 60 min, when [^{3}H]-BK itself was no longer available as a substrate (Figure 3a).

During application of 1,10-phenanthroline, which was the only substance found to inhibit markedly the kininase activity of intact epithelium, the rapid phase of [1-7]-BK and [1-5]-BK generation occurring within the first 30 min was abolished, as was the metabolism of [1-3]-BK to [2-3]-BK and proline (Figure 3b). After an initial lag phase, kininases then became active which slowly produced [1-7]-BK and, to a lesser extent, [1-5]-BK and [1-3]-BK (Figure 3b).

In these studies which also included analysis of small [^{3}H]-BK degradation products, the total amount of radioactivity which could be assigned to specific BK fragments decreased by 7% during the first 10 min of incubation and remained nearly constant thereafter. This virtual complete recovery confirmed that no significant metabolites were excluded from the analysis.

In order to obtain more information on the location of the epithelial kininases, we investigated whether kininase activity remained exclusively in the tissue or whether it was also released into the supernatant. Aliquots of incubation medium were removed from BK-free incubations of intact tracheal epithelium, supplemented with [^{3}H]-BK and further incubated in the absence of tissue. When tracheal epithelium was incubated for less than 30 min, kininase activities in the supernatants were quite low (rate constant $0.014\,min^{-1}$ in supernatant taken after 5 min of incubation). After a 30 min lag phase kininase activities in the supernatants increased steadily reaching a maximum of $0.9\,min^{-1}$ in medium taken after 180 min of incubation (Figure 4). This value corresponded to less than 1% of the [^{3}H]-BK which remained intact after 6 min contact with cell-free tracheal supernatant. A comparison of the metabolite spectra produced by intact epithelium or cell-free supernatants at conditions allowing about 50% degradation of [^{3}H]-BK (30 min incubation with intact epithelium vs. 6 min incubation with supernatant taken after 60 min) revealed very similar patterns (Figure 5).

Degradation of [^{3}H]-BK by isolated epithelial cells

Isolated epithelial cells could be obtained with high viability (about 95% as judged by trypan blue exclusion) in sterile suspensions. Long-term incubation was well tolerated by the cells because they were easily maintained in culture. The epithelial origin was demonstrated by complete staining of cultured cells with anti-cytokeratin. The specificity of the histological method for epithelial cells of trachea has been verified by an exclusive staining of the epithelium in thin slices of this tissue. Freshly isolated epithelial cells were suspended at a protein concentration of $578 \pm 32\ \mu g\ ml^{-1}$ which corresponded to $365 \pm 17\ \mu g$ per 10^6 cells. During incubations with isolated epithelial cells, concentrations of [^{3}H]-BK rapidly decreased in an early phase and more slowly thereafter (Figure 6). The loss of [^{3}H]-BK during the first 10 min was not accompanied by a significant production of metabolites, leading to an initial disappearance of 17% of the total amount of radioactivity detected in all peptide fractions analyzed (Figure 7a). A similar initial loss of radioactivity (13.4% reduction after 5 min) was observed when [^{3}H]-BK was incubated under the same conditions, but in the absence of cells (data not depicted).

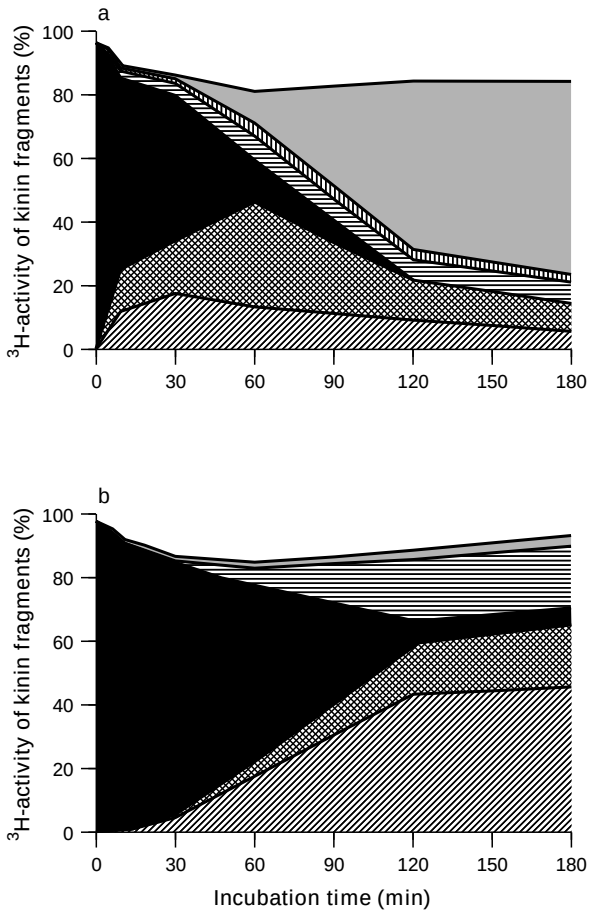

Figure 3 Degradation products of [^{3}H]-bradykinin ([^{3}H]-BK) generated by intact tracheal epithelium. The luminal side of intact epithelium was either incubated (a) without peptidase inhibitors or (b) pretreated (15 min) and incubated in the presence of 1,10-phenanthroline (1 mM). Cumulative presentation of the relative amounts of all peptides detected (solid area, BK; diagonally-hatched area [1-7]-BK; cross-hatched area, [1-5]-BK; horizontally-hatched area, [1-3]-BK; vertically-hatched area, [2-3]-BK and stippled area, proline) in aliquots of the supernatants at the respective incubation times. Data are means of 3 (control) and 2 (phenanthroline) experiments.

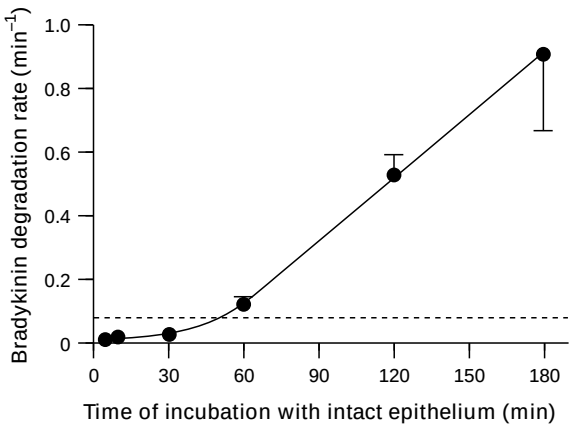

Figure 4 Kininase activity accumulating in the incubation medium of intact tracheal epithelium. Aliquots of supernatant were removed at various timepoints from bradykinin-free incubations of intact epithelium, supplemented with [^{3}H]-bradykinin ([^{3}H]-BK) and further incubated in the absence of tissue for determination of rate constants of [^{3}H]-BK degradation. Kininase activities in the incubation media increased with prolonged exposure to epithelium. The rate constant of [^{3}H]-BK degradation found on intact epithelium itself is indicated in the diagram by a broken line. Kininase activity of intact epithelium = 0.081 min^{-1}. Data are means of 4–5 experiments, vertical lines show s.e.mean.

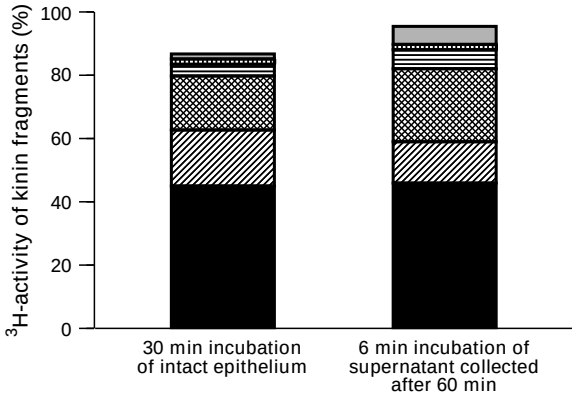

Figure 5 Comparison of [^{3}H]-bradykinin ([^{3}H]-BK) metabolites generated by intact epithelium or cell-free epithelial supernatants. Cumulative presentation of the relative amounts of all peptides detected (solid area, BK; diagonally-hatched area, [1-7]-BK, cross-hatched area, [1-5]-BK; horizontally-hatched area, [1-3]-BK; vertically-hatched area, [2-3]-BK and stippled area, proline). Intact tracheal epithelium was incubated with [^{3}H]-BK for 30 min. Supernatants were removed after 60 min BK-free incubation with intact tracheal epithelium and further incubated for 6 min in the presence of [^{3}H]-BK. Under those conditions comparable amounts of [^{3}H]-BK were degraded in both preparations and the distributions of kinin fragments revealed great similarities of the metabolic pathways. Data are means of 4 (supernatant) and 3 (epithelium) experiments.

As was seen in intact epithelium, [1-7]-BK and [1-5]-BK were major constituents of the metabolite spectrum in suspensions of isolated cells (Figure 7a). In this preparation, however, an additional instantaneous generation of [1-3]-BK and [2-3]-BK was evident. The sum of [1-3]-BK, [2-3]-BK and proline which, as mentioned above, should reflect the initial generation of [1-3]-BK increased in parallel with the amounts of [1-7]-BK and [1-5]-BK (Figure 7a). Since the levels of all metabolites remained constant at the end of the incubations, this suggests that only a very slow conversion of [1-5]-BK or [1-7]-BK to [1-3]-BK took place. Production of [2-3]-BK (Pro-Pro) which was not further degraded to single proline, was another characteristic not observed with the experiments on intact epithelium (Figure 7a).

Use of peptidase inhibitors in suspensions of isolated cells revealed that the rate constant of the rapid initial loss of [^{3}H]-BK was not influenced by any treatment (rate constants of 0.14, 0.145, 0.134 min^{-1} in control, ramiprilat, phosphoramidon groups, respectively) (Figure 6). However, the slow phase of degradation (0.01 ± 0.001 min^{-1} in controls) was significantly inhibited by phosphoramidon (0.0015 ± 0.0004 min^{-1}, $P<0.05$), but not by ramiprilat (0.013 ± 0.002 min^{-1}) (Figure 6). Phosphoramidon exerted two distinct effects on the spectrum of [^{3}H]-BK metabolites (Figure 7b). Firstly, as expected for an inhibitor of NEP, the initial rate of [1-7]-BK formation and the relative contribution of [1-7]-BK to the total amount of kinin fragments was decreased. Secondly, phosphoramidon effectively reduced the generation of [1-3]-BK and [2-3]-BK (Figure 7b). Both inhibitory effects appear to contribute to the reduction of [^{3}H]-BK degradation rate achieved with phosphoramidon.

Discussion

A survey of the various kininases addressed in this study demonstrating the specific sites of direct [^{3}H]-BK cleavage is depicted in Figure 8. Due to the localization of the labelled prolines at positions 2 and 3 of [^{3}H]-BK, only the amino-

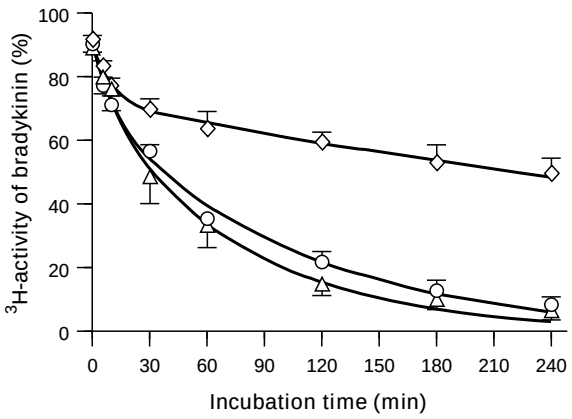

Figure 6 Kinetics of [^{3}H]-bradykinin ([^{3}H]-BK) breakdown in suspensions of freshly isolated tracheal epithelial cells. Time courses of the relative amounts of [^{3}H]-BK in the incubation media under control conditions (○), and after application of ramiprilat (△, 0.25 μM) or phosphoramidon (◇, 10 μM). Time courses were fitted to biexponential dependencies. Rate constants of the rapid phase of [^{3}H]-BK decrease were not different between the treatment groups. The slow phase of [^{3}H]-BK decrease was accompanied by the generation of [^{3}H]-BK metabolites and was significantly inhibited by phosphoramidon (rate constant $0.0015 \pm 0.0004\,\text{min}^{-1}$ vs. $0.01 \pm 0.001\,\text{min}^{-1}$ (control), $P < 0.05$ vs. control). Each point represents the mean of 4 determinations; vertical lines show s.e.mean.

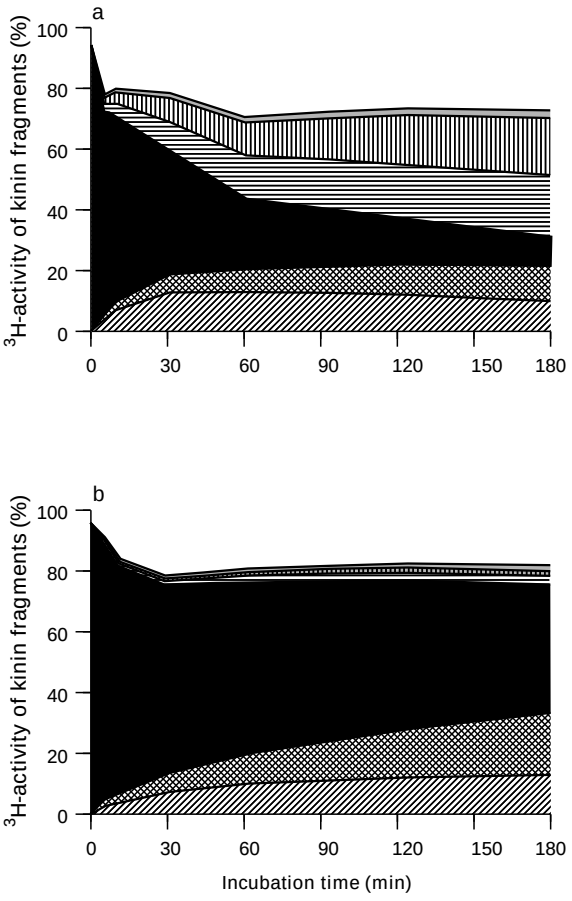

Figure 7 Degradation products of [^{3}H]-bradykinin ([^{3}H]-BK) generated by suspensions of freshly isolated tracheal epithelial cells. Cells were either incubated (a) without peptidase inhibitors or (b) pretreated (15 min) and incubated in the presence of phosphoramidon (10 μM). Cumulative presentation of the relative amounts of all products of [^{3}H]-BK degradation detected (solid area, BK; diagonally-hatched area, [1-7]-BK; cross-hatched area, [1-5]-BK; horizontally-hatched area, [1-3]-BK; vertically-hatched area, [2-3]-BK and stippled area, proline) in aliquots of cell suspensions at the respective incubation times. Data are means of 4 experiments.

terminal products of [^{3}H]-BK cleavage could be detected in the present study.

Identification of peptidases on intact trachea

The most important finding of this study was that neither ACE nor NEP are involved significantly in the degradation of [^{3}H]-BK by bovine intact tracheal epithelium, although the fragments [1-7]-BK and [1-5]-BK, which are specifically generated by these enzymes, are the predominant metabolites formed. This is related only to kinin degradation on the luminal side of epithelium, since the intact epithelium appears to be impermeable to kinins and even to single amino acids like proline.

Taking into consideration only the currently recognized kininases, the generation of [1-7]-BK by intact epithelium can be explained either by sequential cleavage of [^{3}H]-BK by carboxypeptidases or by the activity of dipeptidases like ACE, NEP or PPCE. The first possibility would require formation of [1-8]-BK by a carboxypeptidase type M or N and its subsequent cleavage by prolylcarboxypeptidase. This mechanism appears unlikely since [1-8]-BK was never detected in any of these experiments. Furthermore, functional studies demonstrated no effects of carboxypeptidase M inhibition on bronchoconstriction induced by BK after intraluminal application (Ichinose & Barnes, 1990). The demonstration of carboxypeptidase M in lung tissue by Nagae *et al.* (1993) does not contradict our results in tracheal epithelium since the enzyme has been located at type I alveolar cells specifically. For this reason, direct generation of [1-7]-BK from [^{3}H]-BK by ACE, NEP or PPCE may be assumed. As ACE and NEP inhibitors were ineffective, PPCE remains the only enzyme which can be responsible for the observed metabolism. PPCE is a soluble enzyme widely distributed in human tissues including lung (Kato *et al.*, 1980; Zolfaghari *et al.*, 1986), and is known to cleave BK directly at the positions 3–4 and 7–8, thereby producing [1-3]-BK and [1-7]-BK (Zolfaghari *et al.*, 1986).

The immediate generation of [1-5]-BK on intact epithelium cannot be explained by the activity of PPCE only. Most probably [1-5]-BK was derived from [^{3}H]-BK directly since ACE, the only peptidase known to cleave [1-7]-BK to [1-5]-BK, was not found to be substantially involved in [1-5]-BK generation. Cleavage of [^{3}H]-BK at position 5-6 indicates the presence of MEP which is the only kininase known to have the appropriate specificity. MEP, like PPCE, is a soluble enzyme which has been characterized for the most part in rat brain (Orlowski *et al.*, 1983), although its presence has also been demonstrated in rat trachea and lung tissue (Choi *et al.*, 1990) and guinea-pig trachea (De Silva *et al.*, 1992).

The pathway leading to [1-3]-BK generation could not be conclusively defined. Clearly, [1-3]-BK can be generated from [1-7]-BK or, more likely, [1-5]-BK as precursors. However, direct generation from [^{3}H]-BK may also take place since the delayed production of [1-3]-BK and its metabolites on intact epithelium does not necessarily reflect a second-step generation, but may also be caused by increased kininase activities brought about by a gradual accumulation of enzymes in the supernatant. In any case, PPCE is the only enzyme known to release the fragment [1-3]-BK from BK (Zolfaghari *et al.*, 1986) and its activity on [1-5]-BK and [1-7]-BK has not yet been elucidated.

Involvement of aminopeptidases or carboxypeptidases in directly cleaving [^{3}H]-BK can be excluded solely on the basis of metabolite spectrum analysis, since their direct products, [2-9]-BK or [1-8]-BK, were never detected.

The assumption that PPCE and MEP are the most important kininases on bovine tracheal epithelium is substantiated by the pronounced inhibitory effect of the metalloprotease inhibitor phenanthroline which inhibits MEP completely and PPCE by 48% (Orlowski *et al.*, 1983; Zolfaghari *et al.*, 1986) at the concentration used in this study (1 mM). Under those conditions [^{3}H]-BK degradation only proceeded at incubation times when substantial amounts of

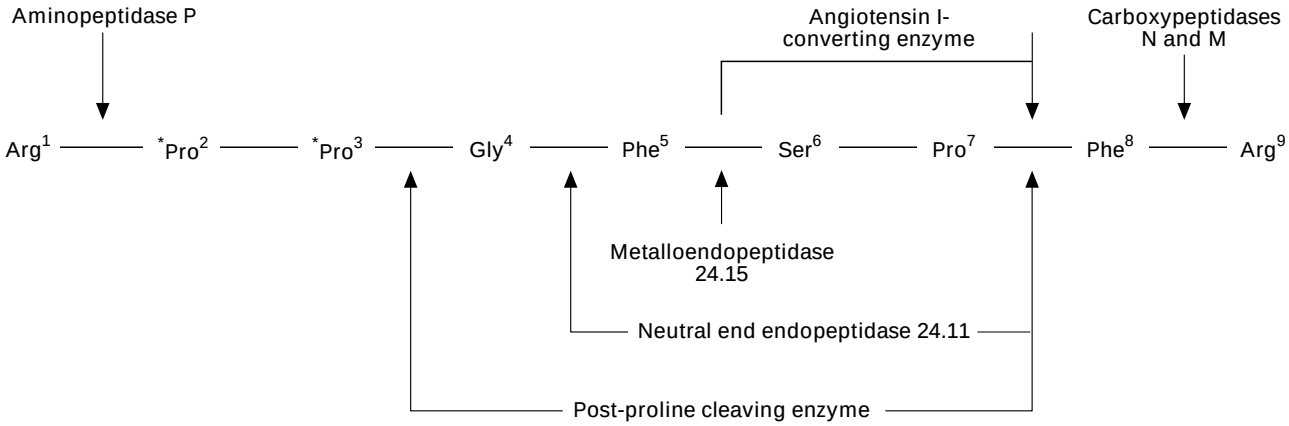

Figure 8 Schematic diagram of the specific sites of bradykinin cleavage by various kininases. Only direct cleavage of intact bradykinin is indicated, with the exception of angiotensin I-converting enzyme which further degrades [1-7]-bradykinin to [1-5]-bradykinin. The kininases listed above the bradykinin sequence were not found to be involved in epithelial bradykinin degradation. The positions of the tritium-labelled proline constituents of the substrate are indicated by asterisks(*).

enzymes had been released from the epithelium and the products appearing (mostly [1-7]-BK and [1-3]-BK) can well be attributed to a residual activity of PPCE.

Another significant finding was that of the continuous release of kininases with very high activity by tracheal epithelium. The uniformity of this effect in all incubations indicates that this activity was due to secretion rather than to a non-specific release induced by cell damage. Epithelial cells even tolerated incubation conditions much harsher than those applied during kinin degradation studies when they were protease-treated for 18 h in order to obtain cell suspensions. Enzymes contained in the epithelial supernatant were not further characterized by inhibitor studies, but the spectra of kinin metabolites generated were largely identical to those of intact epithelium. It was therefore assumed that PPCE and MEP, the two kininases present in intact epithelium, were also released into the incubation medium. The characterization of PPCE and MEP as soluble intracellular enzymes would also be consistent with a proposed localization of these enzymes in secretory granules.

In view of enzymes being actively secreted by the epithelium, a significant part of kininase activity which is detectable at the luminal side of intact epithelium may even be derived from this process. The characterization of kininases as 'epithelium-bound' is based on their presence at the epithelium after a thorough washing procedure, but it is conceivable that secreted peptidases cannot be removed by washing if they are enclosed in tightly bound mucus or retained in gland tubules.

Identification of peptidases from isolated epithelial cells

In suspensions of isolated epithelial cells only the slow phase of [^{3}H]-BK decrease could be attributed to kinin degradation since it proceeded with a concomitant production of metabolites. The early, fast phase of [^{3}H]-BK decrease reflected a reduced recovery for the sum of kinin fragments independent of the presence of cells or inhibitors, and could be explained solely by adsorption of peptides to the incubation wells, as was demonstrated in cell-free control experiments. A comparable adsorption of kinins was not observed in experiments with intact epithelium since in this setup the incubation medium had only contact with a small rim of the incubation wells, whereas the hydrophilic bottoms of the dishes constituted a much larger adsorption area for kinins in experiments with cell suspensions.

Compared to intact epithelium, isolated epithelial cells produce a similar spectrum of metabolites from [^{3}H]-BK, but lack the ability to degrade [1-3]-BK or [2-3]-BK further to proline. The pronounced inhibitory effect of phosphoramidon (85% inhibition), confirms that isolated epithelial cells contain NEP which is responsible for the generation of a significant amount of [1-7]-BK and virtually all the [1-3]-BK. A residual production of [1-7]-BK and [1-5]-BK after application of phosphoramidon can be explained by the low activity of the other tracheal kininases, PPCE and MEP. The nearly exclusive role of NEP in the production of [1-3]-BK might not be due to direct generation of this peptide by NEP, but rather to a direct cleavage between positions 4 and 5 (Zolfaghari *et al*., 1989), leading to a fragment [1-4]-BK which acts as the precursor of [1-3]-BK. However, [1-4]-BK has not been analysed in experiments in which cells suspensions were used and this therefore remains hypothetical. The fact that the sum of all kinin fragments recovered in incubations of epithelial cell suspensions was lower without rather than with phosphoramidon may have reflected a generation of [1-4]-BK as an intermediate product.

Quantitative comparison of both preparations

In contrast to intact epithelium, isolated epithelial cells contain NEP but possess only low active concentrations of other kininases (rate constant of BK breakdown is 0.0015 min^{-1} in the presence of phosphoramidon). Considering that [^{3}H]-BK degradation independent of NEP was largely linked to a probable secretion of enzymes by intact epithelium, the latter finding can be due to destruction of glandular structures or removal of secretory cells during the dissociation procedure. Whereas a decrease of enzyme activity can be easily explained by such methodological factors, the appearance of a new enzyme type requires us to ask whether this activity was actually added or whether it has been uncovered by the removal of other, more active enzymes. Based on a density of 1.86×10^6 cells having contact with one cm^2 of epithelial surface, a number that was calculated from studies on tangential slices of bovine trachea epithelium, the specific activity for intact epithelium can be calculated to be 0.022 $min^{-1}/10^6$ cells, compared to 0.0063 $min^{-1}/10^6$ cells for isolated epithelial cells in suspension. NEP, therefore, would have contributed to nearly one third of the kininase activity in intact epithelium if all of the enzyme detected in single cells was active under those conditions. With a kininase activity of that magnitude, one should have seen a significant inhibitory effect of phosphoramidon on [^{3}H]-BK degradation in intact epithelium which was not observed. NEP is therefore either evenly distributed in all cell membranes or is concentrated primarily at the basolateral side of the epithelium, so that the major part of the enzyme was newly exposed to [^{3}H]-BK upon tissue dissociation. Indeed, it has been shown that NEP, detected by immunocytochemical means, is preferentially associated with basal cells of the multi-layer airway epithelium (Nadel & Borson, 1991).

Considerations on the potentiation of the effects of BK

In this study the functionally significant kininases were identified as PPCE and MEP at the luminal and as NEP at the basolateral side of tracheal epithelium, while ACE was found to have no quantitative significance. How do these biochemical findings correlate to the potentiation of BK effects by various peptidase inhibitors investigated in previous studies?

Our studies are in good agreement with those of others concerning the important role of NEP for the metabolism of BK in airways. Potentiation by inhibition of NEP has been shown for BK-induced contraction of ferret tracheal rings (Dusser *et al*., 1988), for BK-dependent stimulation of ciliary activity in rabbit cultured tracheal epithelial cells (Tamaoki *et al*., 1989) and for the increase in airway resistance provoked by inhalation of BK in asthmatic patients (Crimi *et al*., 1995).

With respect to ACE, the evidence seems more controversial. Inhibition of ACE had no effect on BK-dependent stimulation of ciliary activity in rabbit cultured tracheal epithelial cells (Tamaoki *et al*., 1989) and on BK-induced increase of airway resistance in patients with asthma or ACE-inhibitor dependent cough (Overlack *et al*., 1992). On the other hand, ACE inhibitors potentiated BK effects in studies with abluminal application of BK, e.g. *in vitro* contraction of ferret tracheal rings (Dusser *et al*., 1988), or when vascular effects of BK were evaluated, such as BK-induced vascular leakage in bronchi of ventilated guinea pigs (Lötvall *et al*., 1991). Vascular leakage might also have been the reason for BK-induced increase of airway opening pressure being enhanced by inhibition of ACE or NEP in a similar experimental model (Ichinose & Barnes, 1990). The latter investigators also excluded a role of carboxypeptidases in the metabolism of BK. The inconsistent findings with respect to ACE may reflect the exclusively vascular localisation of this enzyme which makes the evaluation of its functional importance very much dependent on the route of BK application and the nature of effect measured.

The involvement of MEP in the degradation of BK was demonstrated in guinea-pig trachea by use of a specific inhibitor which potentiated contraction (Da Silva *et al*., 1992). However, there is no further indication of the functional importance of MEP or PPCE or additional secreted kininases in other models. As an example that proteases can be released by tracheal epithelium, secretion of gelatinase A from tracheal gland serous cells has been demonstrated (Tournier *et al*., 1994). However, no information exists for the involvement of this enzyme in kinin degradation.

With regard to the significance of all functional studies one has to bear in mind that the degradation pathways discussed are not specific for kinins so that BK-mediated effects can be influenced indirectly. For example, BK stimulates release of substance P amongst other tachykinins (Saria *et al*., 1988) which, like BK, can be metabolized by NEP and ACE (Skidgel *et al*., 1984) and thus may contribute to the actions of the respective enzyme inhibitors.

In conclusion, this study demonstrates that at the luminal surface of the bovine tracheal epithelium ACE and NEP are not detectably involved in the degradation of [^{3}H]-BK. The metabolism of [^{3}H]-BK on intact epithelium proceeds through a pathway which is consistent with the combined activities of MEP and PPCE. However, the identification of these enzymes is deduced from the spectrum of kinin metabolites generated and the existence of a novel kind of kininase has not been excluded. Large amounts of these kininases are secreted by the epithelium. The epithelium is impermeable to kinins and kinin fragments and, if intact, should be able to prevent direct effects of luminal BK on the tracheal wall. At the basolateral side of the epithelium NEP is the predominant kininase present. In this location it may act to protect the organ against bronchoconstriction which might otherwise be induced by BK in the event that the integrity of the tracheal epithelium becomes disrupted.

These results would not seem to suggest an important role for BK in ACE-inhibitor induced cough, if data from bovine trachea are transferable in this respect. Preservation of the integrity of tracheal epithelium appears to be a promising target for future pharmacotherapy. The nature and physiological roles of secreted kininases need further investigation.

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