Cathepsin K: a cysteine protease with unique kinin-degrading properties

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Taking into account a previous report of an unidentified enzyme from macrophages acting as a kininase, the ability of cysteine proteases to degrade kinins has been investigated. Wild-type fibroblast lysates from mice, by contrast with cathepsin K-deficient lysates, hydrolysed BK (bradykinin), and released two metabolites, BK-(1–4) and BK-(5–9). Cathepsin K, but not cathepsins B, H, L and S, cleaved kinins at the Gly^4–Phe^5 bond and the bradykinin-mimicking substrate Abz (o-aminobenzoic acid)-RP-PGFSPFR-3-NO$_2$-Tyr (3-nitrotyrosine) more efficiently (pH 6.0: $k_{cat}/K_m = 12\,500$ mM$^{-1} \cdot$ s^{-1}; pH 7.4: $k_{cat}/K_m = 6930$ mM$^{-1} \cdot$ s^{-1}) than angiotensin-converting enzyme hydrolysed BK. Conversely Abz-RPPGFSPFR-3-NO$_2$-Tyr was not cleaved by the Y67L (Tyr$^{67} \rightarrow$ Leu)/L205A (Leu$^{205} \rightarrow$ Ala) cathepsin K mutant, indicating that kinin degradation mostly depends on the S2 substrate specificity. Kininase activity was further evaluated on bronchial smooth muscles. BK, but not its metabolites BK(1-4) and BK(5-9), induced a dose-dependent contraction, which was abolished by Hoe140, a B2-type receptor antagonist. Cathepsin K impaired BK-dependent contraction of normal and chronic hypoxic rats, whereas cathepsins B and L did not. Taking together vasoactive properties of kinins and the potency of cathepsin K to modulate BK-dependent contraction of smooth muscles, the present data support the notion that cathepsin K may act as a kininase, a unique property among mammalian cysteine proteases.

Key words: cathepsin, cysteine protease, kinin, kininase, lung inflammation.

INTRODUCTION

Owing to the strong proinflammatory properties of kinins resulting from plasma extravasation, activation of mast cells, fibroblasts and macrophages, and the release of inflammatory mediators (NO, prostaglandins, leukotrienes and cytokines), kinins are believed to play an important role in a variety of lung inflammatory diseases [1]. Asthma, which is characterized by repeated episodes of airflow obstruction [2], and chronic obstructive pulmonary disease display many similarities, including bronchoconstriction and AHR (airway hyper-responsiveness) [3]. Kinins [e.g. BK (bradykinin), and related kinins such as lysyl-bradykinin, also called kallidin], whose pharmacological effects are mediated by BK receptors, are peptide hormones that participate in the maintenance of cardiovascular homoeostasis (for a review, see [4]). It has been shown that, after an inflammatory challenge, lung kinin-releasing activity is increased in nasal washings and broncho-alveolar fluids [5]. Furthermore BK, which induces bronchoconstriction and AHR in asthmatic patients, has been proposed as a mediator of asthma [6]. Conversely, blockade of BK receptors by specific antagonists significantly reduced bronchoconstriction and AHR of patients suffering from perennial rhinitis and asthma [1].

In inflamed lung tissues, enhanced protein breakdown occurs as a result of protease/antiprotease imbalance, and may cause severe damage [7,8]. This process is accompanied by the release, from macrophages at the site of injury, of thiol-dependent cathepsins that participate in the spread of inflammation [9,10]. Furthermore, the activity of CPs (cysteine proteases) is favoured by the acidification of the pericellular environment of macrophages, via increased expression of an H$^+$-ATPase pump [11,12]. Besides their housekeeping tasks (intracellular protein degradation and turnover) within the endosomal/lysosomal system, mammalian cathepsins B, K, L and S fulfil more specific physiological functions in MHC-II antigen presentation or in prohormone processing [13]. They are also involved in pathological processes such as Alzheimer's disease, tumour invasion, muscular dystrophy, osteoporosis or rheumatoid arthritis (for a review, see [14]). Among mammalian CPs, cathepsin K, which is predominantly expressed in osteoclasts, exhibits a unique chondroitin sulphate-dependent collagenolytic activity [15,16]. Cathepsin K has been also found in the serum of Gaucher patients, in lung epithelial cells, in cancer prostate cells, in macrophages, and has been proposed as a marker of inflammation [17–20]. During our preliminary efforts to characterize proteolytic activities of CPs from human inflammatory bronchoalveolar lavage fluids (C. Serveau-Avesque, M. Ferrer-Di Martino, and G. Lalmanach, unpublished work), we have observed that cathepsin K may hydrolyse BK at acidic pH [21]. On the other hand, p-CMPSA (p-chloromercuriphenylsulphonic acid), a thiol-group-blocking reagent, significantly delayed the breakdown of BK by macrophages, by inactivation of an

unidentified kininase [22], that is not related to well-documented kininases such as neutral endopeptidase, carboxypeptidase N or ACE (angiotensin-converting enzyme) (for reviews, see [23,24]). In addition, evidence has been provided for the presence of potentially new enzymes and mechanisms of BK degradation [25]. Taken together, these data have led us to investigate the capacity of cathepsin K to act as a kininase. According to this hypothesis, kinetics studies using kinins and/or synthetic kinin-mimicking peptides were performed in order to characterize the kinin-degrading properties of cathepsin K. Moreover, the ability of cathepsin K to modulate the vasoactive properties of BK and its subsequent effects on BK receptors of isolated rat bronchial smooth muscles was investigated. The present report supports our hypothesis that cathepsin K, by impairing the BK-B_2-receptor-mediated contraction phase, may be a new kininase of physiological relevance.

EXPERIMENTAL

Materials

E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane], PMSF, pepstatin, chondroitin 4-sulphate, Hoe140 (a B2-type receptor antagonist) and des-Arg[10]-Hoe140 were obtained from Sigma–Aldrich (St Quentin le Fallavier, France). Captopril and Mergepta [MGTA (DL-2-mercaptomethyl-3-guanidinoethylthio-propanoic acid)] were supplied by Calbiochem (VWR International, Pessac, France). Z (benzyloxycarbonyl)-Phe-Arg-AMC (7-amino-4-methylcoumarin hydrochloride) and DTT (dithiothreitol) were purchased from Bachem (Weil am Rhein, Germany). All other reagents were of analytical grade.

Enzymes

Human cathepsins B, L and S were supplied by Calbiochem. Wild-type human cathepsin K and the Y67L (Tyr[67] → Leu)/L205A (Leu[205] → Ala) cathepsin K mutant were expressed in *Pichia pastoris* as described elsewhere [26]. Cathepsins were activated in 0.1 M phosphate buffer, pH 6.0, containing 2 mM DTT and 1 mM EDTA, for 5 min at 37 °C prior to kinetic measurements with a Kontron SFM 25 spectrofluorimeter. Their active sites were titrated with E-64 [27], using Z-Phe-Arg-AMC as substrate (excitation wavelength 350 nm; emission wavelength 460 nm). Human tissue kallikrein was obtained from Sigma–Aldrich, whereas bovine pancreatic trypsin was purchased from Boehringer (Roche Molecular Biochemicals, Mannheim, Germany). The buffer for trypsin was 0.1 M Tris/HCl. pH 8.5, containing 0.15 M NaCl, and that for hK1 (human tissue kallikrein) was 50 mM Tris/HCl, pH 8.3, containing 1 mM EDTA. Trypsin and hK1 were titrated as reported elsewhere [28].

Peptides

All Fmoc (fluoren-9-ylmethoxycarbonyl)-protected amino acids were of the L configuration, and were purchased from Neosystem (Strasbourg, France), Novabiochem (VWR international) and Advanced Chemtech (Cambridge, U.K.). The intramolecularly quenched fluorogenic substrate Abz (*o*-aminobenzoic acid)-RPPGFSPFR-(3-NO$_2$-Tyr), also called Abz-BK-(3-NO$_2$-Tyr), was prepared by Fmoc chemistry on an automated solid-phase peptide synthesizer (The Pioneer; Applied Biosystems, Warrington, U.K.), using a PAL-PEG-PS resin (Applied Biosystems). After removal of the sidechain protecting groups and cleavage from the resin, the peptidyl amide Abz-RPPGFSPFR-3-NO$_2$-Tyr was purified by semipreparative reverse-phase chromatography (Vydac C$_{18}$ 218TPS1 column), using a 35 min linear

(0–60 %) gradient of acetonitrile in 0.1 % TFA (trifluoroacetic acid). Finally, Abz-RPPGFSPFR-3-NO$_2$-Tyr was checked for homogeneity by analytical reverse-phase HPLC (Brownlee C$_{18}$ OD 300 column), using the elution conditions indicated above, and their molecular masses checked by MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) (Biflex III; Brüker, Wissembourg, France) or ESI-Q-TOF (electrospray ionization–quadrupole–time-of-flight) MS (Micromass, Birmingham, U.K.). BK and des-Arg[9]-BK were obtained from Sigma–Aldrich. Kallidin (Lys-BK) was from Advanced ChemTech (Louisville, KY, U.S.A.). The BK-derived pentapeptidylamide BK-(5–9) (Phe-Ser-Pro-Phe-Arg) was prepared as decribed in [21]. BK-(1–4) (Arg-Pro-Pro-Gly) was made by a similar procedure, using tetramethylfluoroformamidium hexafluorophosphate as activator and a Novasyn TGR resin (Novabiochem).

Kininase activity of cathepsin K

BK, Lys-BK and des-Arg[9]-BK were incubated with cathepsin K (enzyme/substrate molar ratio = 1:2500) for 0–120 min at 37 °C in 0.1 M phosphate buffer, pH 6.0, containing 2 mM DTT and 1 mM EDTA and the products analysed by reverse-phase chromatography [C$_{18}$ Brownlee ODS-032 column; 45-min linear (0–60 %) gradient of acetonitrile in 0.1 % trifluoroacetic acid]. Kinin metabolites were quantified by running the ChromQuest Chromatography Workstation (ThermoFinnigan, les Ulis, France), and were identified by N-terminal peptide sequencing (Procise sequencer; Applied Biosystems). Similar experiments were performed with cathepsins B, H, L, S and hK1. The same procedure was repeated in 0.1 M phosphate buffer, pH 7.4, containing 2 mM DTT and 1 mM EDTA.

The second-order rate constants for the hydrolysis of 0.5 μM Abz-RPPGFSPFR-3-NO$_2$-Tyr by cathepsins B, L, S and K by the Y67L/L205A mutant, by trypsin and by hK1, were determined under pseudo-first-order conditions (excitation wavelength 320 nm; emission wavelength 420 nm). Calibration was performed as described elsewhere [29]. Triplicate assays (except for cathepsin K, n = 10) were performed in the presence of cathepsins K (1–4 nM), B (30 nM), S (20 nM), L (20 nM), Y67L/L205A cathepsin K (20 nM), trypsin (20 nM) or hK1 (20 nM) in their respective activity buffer at 37 °C. Kinetic data were determined using the Enzfitter software (Biosoft, Cambridge, U.K.) and are reported as means ± S.D. Michaelis (K_m) and catalytic (k_{cat}) constants for the hydrolysis of Abz-BK-3-NO$_2$-Tyr (0.05–2 μM) by cathepsin K were determined by non-linear regression analysis.

Cathepsin K (3 nM) was incubated with Abz-BK-3-NO$_2$-Tyr (8 μM) for 3 min at 37 °C in its assay buffer (final volume 200 μl), and the reaction stopped by adding 800 μl of ethanol. After removal of the precipitate, the supernatant containing the native peptide and/or its proteolytic fragments was evaporated to dryness and re-dissolved in 0.1 % TFA. An aliquot of each sample was fractionated by reverse-phase HPLC on a C$_{18}$ OD 300 column (Brownlee) using a 30 min linear (0–60 %) gradient of acetonitrile in 0.1 % TFA at a flow rate of 0.3 ml/min. Proteolysis products were analysed by running the ChromQuest Chromatography Workstation, and cleavage sites were located by N-terminal sequencing. The same protocol was repeated for hK1(70 nM), trypsin (100 nM) and cathepsin L (70 nM).

Cathepsin K activity assays on cultured fibroblasts

Cathepsin K knockout (cat K$^{-/-}$) mice (C57BL/6J background) were generated by targeted disruption of the cathepsin K gene [30]. Skin fibroblasts from wild-type and cathepsin K-deficient mice were cultured as previously described [31]. Finally, cell lysates were obtained in 100 mM sodium acetate buffer, pH 5.5,

2.5 mM DTT, 2.5 mM EDTA, 10 μM PMSF and 10 μM pepstatin A. After centrifugation (13 000 g for 10 min at 4 °C), the protein concentrations in the supernatants of wild-type and knockout cells were determined using the Bradford kit (Bio-Rad, Hercules, CA, U.S.A.). Western-blot analysis was performed using a rabbit polyclonal antibody directed against mouse cathepsin K [31].

After activation for 2 min in 0.1 M phosphate buffer, pH 6.0, containing 2 mM DTT, 1 mM EDTA, 0.01 % Brij 35, 0.04 mM pepstatin A and 0.4 mM PMSF, supernatants (5 μg of total protein/assay) were incubated with BK (16.6 μM) for 6 h at 30 °C. After removal of proteins by ethanol, peptidyl fractions containing native BK and/or its hydrolysis products were separated by reverse-phase chromatography [C_{18} Merck LiChroCart Cartridge; 20 min linear (0–60 %) gradient of acetonitrile in 0.1 % TFA], and identified by N-terminal sequencing (Procise sequencer) and ESI-Q-TOF MS. The same procedure was repeated in the presence of 10 μM E-64.

Tissue preparation from normal and hypoxic rats

All animal experiments were conducted according to the ethical standards of the Ministère Français de l'Agriculture. On the day of experiments, Wistar rats were killed with sodium pentobarbital (60 mg/kg intraperitoneally). The thoraxes were opened and lungs quickly removed and immerged in cold (4 °C) physiological saline solution (PSS) containing 138.6 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl$_2$, 1.2 mM MgCl$_2$, 0.33 mM NaH$_2$PO$_4$, 10 mM Hepes and 11 mM glucose (the pH was adjusted to 7.4 using NaOH). The bronchi were then rapidly isolated and gently cleaned of parenchyma and adhering connective tissue under a dissection microscope. The bronchi were cut in rings 3 mm long and the epithelium was kept intact. Conversely a chronically hypoxic rat model was developed as previously described [32]. To summarize, the rats were set under chronic-hypoxia conditions by decreasing the atmospheric pressure to 50.5 kPa ($pO_2 = 10$ kPa) for 21 days. Bronchi were isolated and prepared for isometric measurements as described above.

Recording of mechanical activity

Mechanical responses were measured as previously described [33]. Briefly, isolated rings were attached between a rigid support and the arm of a force transducer (Pioden Controls Ltd., Canterbury, U.K.) by slipping the ring over two stainless-steel wires. One of the wires was anchored to the rigid support and the other was connected to an isometric transducer. The rings were placed in an organ chamber and bubbled at a constant flow rate with a O$_2$/N$_2$ (21:79) gas mixture, then were set at optimal length by equilibration against a passive load. After undergoing a stress relaxation equilibration period of 60 min (to reach the residual passive tension) the isometric contractions were recorded. The rings were precontracted with Ach (acetylcholine) at 10^{-5} M (concentration inducing the maximal contraction) and when the contractile response reached a stable tension, kinin solutions, that is BK, BK-(1–4) and BK-(5–9), were applied (0.001 nM–0.01 mM). For some experiments, a cumulative concentration-response for BK was determined by increasing concentration from 10^{-12} to 10^{-5} M. Contraction amplitudes were expressed in milligrams and were measured from the resting tension. All the data were collected with a computerized data-acquisition system using Genie software (Advantech, Sunnyvale, CA, U.S.A.).

Analysis and statistics

Results are expressed as means $\pm$ S.E.M. The contractions are expressed as percentages of Ach-induced contraction. All the
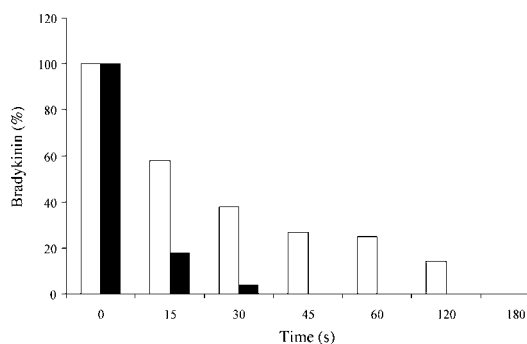

Figure 1   Hydrolysis of BK by cathepsin K

Human cathepsin K was incubated with BK (enzyme/substrate molar ratio 1:2500) at 37 °C, in 0.1 M phosphate buffer, pH 6.0, containing 2 mM DTT and 1 mM EDTA for periods of 0–5 min (black bar). The same procedure was repeated in 0.1 M phosphate buffer, pH 7.4, containing 2 mM DTT and 1 mM EDTA (white bar). Hydrolysis products were further fractionated by reverse-phase HPLC (C_{18} Brownlee ODS-032 column) (see the Experimental section for details). For the sake of clarity, the residual amount of BK was normalized.

analysis was done by using Origin 6 software (Microcal Software, Northampton, MA, U.S.A.). Statistical analysis was done using one-way factor ANOVA followed by Student's t test. Differences were considered significant when $P < 0.05$. Statistical analysis was done using Minitab software (Minitab Inc., State College, PA, U.S.A.). The number of experiments (n) refers to the number of rings.

RESULTS AND DISCUSSION

Specific cleavage of BK by cathepsin K

In our efforts to characterize proteolytic activities of CPs from human inflammatory BAL (bronchoalveolar lavage) fluids, we have explored the ability of cathepsin L-like enzymes to participate in the production of kinins [21,34], and observed that cathepsin K may hydrolyse BK. In the present study, in-depth analysis of the putative kininase activity of cathepsin K was performed. BK, Lys-BK and des-Arg[9]-BK were rapidly degraded by cathepsin K under mildly acidic conditions (pH 6.0) and at pH 7.4 (Figure 1), leading to the release of two stable products: BK-(1–4) (Arg[1]-Pro-Pro-Gly[4]) and BK-(5–9) (Phe[5]-Ser-Pro-Phe-Arg[9]) (enzyme/substrate molar ratio of 1:2500), in agreement with its pH-dependent activity profile. Interestingly neutral endopeptidase degrades BK at the same cleavage site [24]. By contrast, BK, Lys-BK and des-Arg[9]-BK remained uncleaved and stable upon incubation with cathepsins B, L, S and H when subjected to reverse-phase-chromatography analysis (results not shown).

Beside its major well-documented expression in osteoclasts [13], the presence of both procathepsin K and its mature active form has been reported in synovial and skin fibroblasts, as shown by Western-blot analysis of cell lysates from wild-type and cathepsin K-deficient (cat K$^{-/-}$) mice [31]. After 6 h incubation of cell extracts from skin fibroblasts with exogenous BK (16.6 μM) in the presence of a thiol-reducing reagent (2 mM DTT) and protease inhibitors (1 mM EDTA/400 μM PMSF/40 μM pepstatin A), peptidyl fractions were separated by reverse-phase HPLC and analysed by N-terminal sequencing and ESI-Q-TOF MS (Figure 2). Wild-type fibroblast lysates, but not cathepsin K-deficient lysates, hydrolysed BK and released a major proteolysis product whose retention time and molecular mass (experimental molecular mass 653.33 Da) corresponded to BK-(5–9) (theoretical molecular mass 653.34 Da). By contrast, addition of E-64 to
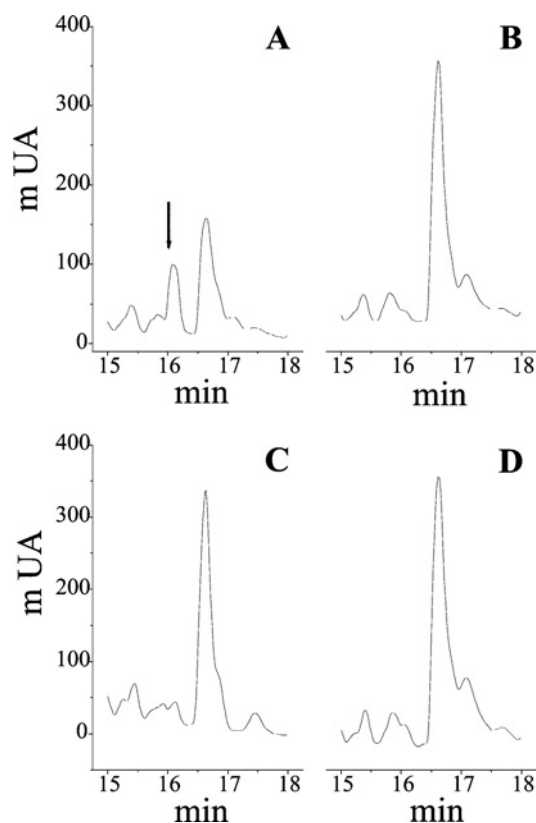
Figure 2   Selective hydrolysis of BK by cathepsin K from skin fibroblasts

Fibroblast lysates from wild-type and cathepsin K-deficient (cat K$^{-/-}$) mice (C57BL/6J background) were prepared as described elsewhere [31]. After activation for 2 min in 0.1 M phosphate buffer, pH 6.0, containing 2 mM DTT and 0.01 % Brij 35, and in presence of 1 mM EDTA, 0.04 mM pepstatin A and 0.4 mM PMSF, supernatants from fibroblast lysates (5 μg total protein/assay) were incubated with BK (0.16 μM) for 6 h at 30 °C. Native BK and/or its hydrolysis products were separated by reverse-phase HPLC, prior identification by N-terminal sequencing and ESI-Q-TOF MS (see the Experimental section). The major hydrolysis product, BK-(5–9), is identified by an arrow. (**A**) Wild-type lysates; (**B**) wild-type lysates + E-64 (10 μM); (**C**) cat K$^{-/-}$ lysates; (**D**) cat K$^{-/-}$ lysates + E-64 (10 μM). Abbreviation: mUA, milli-units of absorbance at 220 nm.

wild-type lysates totally abolished the degradation of BK, suggesting that the kininase activity is indeed catalysed by cathepsin K.

Enzymic activity on a fluorescent BK-mimicking peptide

We further analysed and quantified BK metabolism by cathepsin K using the intramolecularly quenched fluorogenic BK-derived peptidylamide Abz-BK-3-NO$_2$-Tyr, consisting of the nonamer BK moiety flanked by a fluorescent N-terminal donor group (Abz) and a C-terminal quencher (3-NO$_2$-Tyr). The second-order rate constants were measured under pseudo-first-order conditions for all enzymes assayed. In addition, K_m and k_{cat} values were determined by non-linear regression analysis, and the specificity constant (k_{cat}/K_m) deduced from individual K_m and k_{cat} values for cathepsin K. Abz-BK-3-NO$_2$-Tyr was very efficiently hydrolysed by recombinant human cathepsin K with a second-order rate of hydrolysis of 12 500 mM$^{-1} \cdot$ s^{-1} ($K_m = 0.4$ μM, $k_{cat} = 5$ s^{-1}). Similar rate constants were obtained under pseudo-first-order reaction conditions ($k_{cat}/K_m = 10 370$ mM$^{-1} \cdot$ s^{-1}; Table 1). Surprisingly, a significantly lower turnover rate by cathepsin K for a similar BK-derived peptide [Abz-RPPGFSPFR-EDDnp, where EDDnp is N-(2,4-dinitrophenyl)ethylenediamine] was recently reported

Table 1   Kinetic parameters for the hydrolysis of Abz-RPPGFSPFR-3-NO$_2$-Tyr

Experiments were carried out as described in the Experimental section. Kinetic data were determined using Enzfitter software (Biosoft, Cambridge, U.K.) and are reported as means $\pm$ S.D. (triplicate assays; except for cathepsin K, $n = 10$). The Michaelis constant (K_m) and the catalytic constant (k_{cat}) values were determined by non-linear regression analysis.

Enzyme	k_{cat}/K_m (mM$^{-1} \cdot$ s^{-1})*
Cathepsin K	
pH 6.0	10 370 $\pm$ 340
	12 500†
pH 7.4	6930 $\pm$ 680
Cathepsin L	14 $\pm$ 0.4
Cathepsin B	45 $\pm$ 1
Cathepsin S	52 $\pm$ 2
Y67L/L205A‡	5 $\pm$ 0.2
hK1	38 $\pm$ 1
ACE§	5000

* Determined under pseudo-first-order conditions.
† Deduced from individual K_m and k_{cat} values ($K_m = 0.4 \pm 0.07$ μM; $k_{cat} = 5 \pm 0.2$ s^{-1}).
‡ Y67L/L205A cathepsin K mutant [26].
§ From [36], using bradykinin as substrate.

($K_m = 1.7$ μM, $k_{cat} = 0.1$ s^{-1} and $k_{cat}/K_m = 62.8$ mM$^{-1} \cdot$ s^{-1}) [35]. Both substrates are only distinguished by their C-terminal quenching groups, 3-NO$_2$-Tyr versus EDDnp. Presently it can only be speculated whether the observed differences in the rate constants are related to the quenching groups or variation in the assay conditions. Kinetics data support the notion that cathepsin K specificity for BK is comparable with that of angiotensin I-converting enzyme, which inactivates BK by the sequential removal of two C-terminal dipeptides [36]. Furthermore, cathepsin K was still highly efficient at pH 7.4 ($k_{cat}/K_m = 6930$ mM$^{-1} \cdot$ s^{-1}). Similar second-order rate constants (pH 6.0: $k_{cat}/K_m = 10 600$ mM$^{-1} \cdot$ s^{-1}; pH 7.4: $k_{cat}/K_m = 6530$ mM$^{-1} \cdot$ s^{-1}) were obtained in the presence of chondroitin 4-sulphate, a major glycosaminoglycan of the extracellular matrix. While chondroitin 4-sulphate is required for the collagenolytic activity of cathepsin K, present data indicate that its presence is not essential for BK hydrolysis, as previously reported for hydrolysis of small peptide substrates [37]. According to N-terminal sequencing results, cleavage occurred at the Gly^4–Phe^5 bond, as observed with native BK. This is in agreement with the substrate specificity of cathepsin K, since proline and glycine are favourable residues at the P2 and P1 positions respectively [31,35]. On the other hand, cathepsins B, L and S, as well as hK1, only weakly hydrolysed Abz-BK-3-NO$_2$-Tyr, with approximately three orders of magnitude lower efficiency (Table 1). Accordingly, native BK is not a physiological target for cathepsins B, L and S, and hK1 [4]. Analysis of the hydrolysis pattern of Abz-BK-3-NO$_2$-Tyr by hK1 and cathepsin L confirmed the absence of kininase activity, since the cleavage site was located at the amide bond between the C-terminal BK residue and the additional C-terminal fluorescence quencher, i.e. between Arg^9 and (3-NO$_2$-Tyr). This non-physiological cleavage site after Phe^8-Arg^9 is not surprising, since it corresponds to one of the preferred pairs of residues accommodated at the S2 and S1 subsites in trypsin-like enzymes as well as papain-like enzymes [38]. The Y67L/L205A cathepsin K mutant, which displays a S2 cathepsin L-like specificity [26], did not cleave at the Gly^4–Phe^5 bond but poorly hydrolysed Abz-BK-3-NO$_2$-Tyr ($k_{cat}/K_m = 5$ mM$^{-1} \cdot$ s^{-1}) at the Arg^9–3-NO$_2$-Tyr bond, indicating that the kininase activity of cathepsin K critically depends on P2–S2 interactions. Furthermore, cathepsin K hydrolysed Abz-BK-3-NO$_2$-Tyr ($k_{cat}/K_m = 742 \pm 50$ mM$^{-1} \cdot$ s^{-1}) with efficiency in PSS, a
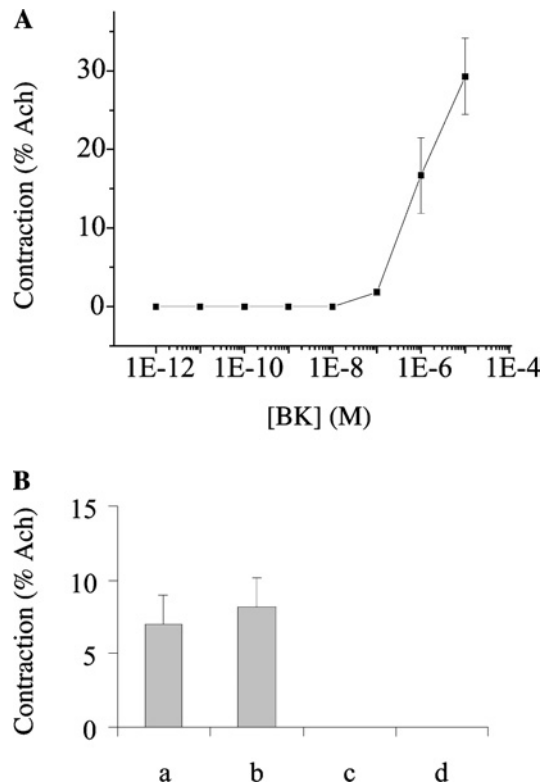
Figure 3   Effect of BK on bronchial smooth muscles from normal rat

(**A**) The dose–response curve was obtained by increasing concentrations of BK. After the initial administration of Ach (10^{-5} M), BK (10^{-12} M–10^{-5} M) was added as described in the Experimental section. (**B**) Effect of antagonists on BK-mediated contraction. Rings of bronchial smooth muscles were pretreated in the presence of des-Arg[10]-Hoe140 (1 μM), a BK B_1 receptor antagonist (b, $n = 8$), Hoe140 (1 μM), a BK B_2 receptor antagonist (c, $n = 4$) or with both antagonists (d, $n = 7$), prior to the addition of 0.5 μM BK. The responses were compared with the control condition (a, $n = 19$). Antagonists were applied 20 min prior to the addition of BK. Results are means $\pm$ S.E.M. Contractions are expressed as percentage of the Ach response.

reducing-agent-free isotonic medium used to record isometric responses of isolated bronchial rings (see the next paragraph), supporting the notion that cathepsin K has the potential to degrade kinins extracellularly.

Effects of cathepsin K on BK-mediated contraction of bronchial smooth muscles

Briefly, contractions of freshly isolated bronchial smooth-muscle rings, placed in an organ chamber filled with PSS and bubbled at a constant flow rate with an O_2/N_2 (21:79) gas mixture, were measured by using an isometric transducer. After precontraction with ACh (10^{-5} M) and stabilization of the contractile response, BK, BK-(1–4) or BK-(5–9) was added (0.001 nM–0.01 mM). In contrast with its metabolites [BK-(1–4) and BK-(5–9)], BK ($\geqslant 10$ nM) induced a dose-dependent contractile response (Figure 3A). A similar BK-dependent contraction was observed in the presence of MGTA and captopril (inhibitors of carboxypeptidase M and ACE respectively), indicating that endogenous kininases [39] did not affect the contractile response of the bronchial smooth muscles under our experimental conditions. Moreover, the BK-dependent response was fully abolished in the presence of Hoe140 (1 μM), but not des-Arg[10]-Hoe140 (1 μM) (Figure 3B), indicating that bronchial smooth-muscle contraction is mediated by constitutive BK B_2-type receptors.

Cathepsin K (1 nM) was added to BK (0.5 μM), and the reaction mixture (40 μl) was immediately applied to the PSS bath solution
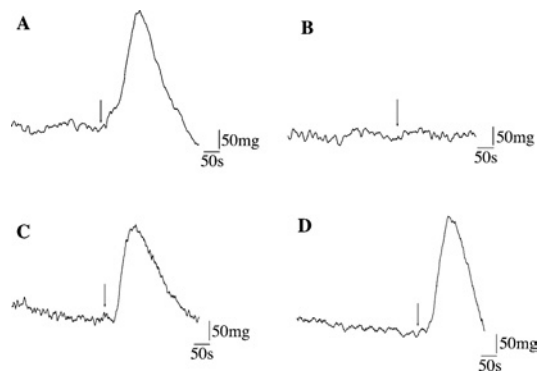

Figure 4   Effect of cathepsins K, B, and L on BK-mediated transient contraction on normal bronchial smooth-muscle rings

Original representative traces showing the effect of cathepsins on BK-dependent contractions are reported: (**A**) E-64-treated cathepsin K (1 nM); (**B**) cathepsin K (1 nM); (**C**) cathepsin B (1 nM); and (**D**) cathepsin L (1 nM). BK (0.5 μM) was mixed with cathepsins (enzyme/substrate molar ratio 1:500) and immediately added (arrow) to the organ bath solution containing PSS.

of the organ chamber (4 ml). Cathepsin K totally abolished contraction of Ach-precontracted rings (Figure 4A), while treatment with E-64 fully restored the BK-induced contractile response (Figure 4B). On the other hand, cathepsins B and L did not impair BK-dependent contraction (Figures 4C and 4D). Removal of an aliquot and analysis of the corresponding peptide supernatant by reverse-phase HPLC confirmed that the loss of contraction was associated with BK hydrolysis and subsequent release of BK-(1–4) and BK-(5–9). This confirms that the ability of cathepsin K to modulate the activity of B_2-receptors depends on its proteolytic activity. The same response was observed in presence of MGTA and captopril, indicating that the kininase activity was not related to endogenous lung carboxypeptidase M and ACE respectively [39]. Along with the kinetics data showing that cathepsin K hydrolysed efficiently Abz-BK-3-NO_2-Tyr in the absence of reducing reagent ($k_{cat}/K_m = 742 \pm 50$ mM$^{-1} \cdot$ s^{-1}), the present results confirmed the unique potency of cathepsin K among mammalian CPs to act as a physiological kininase.

Cathepsin K-impaired BK-mediated contraction of hypoxic rats

Asthma and chronic obstructive pulmonary disease are associated with an inflammation, a hyper-responsiveness and a remodelling of the airways. Taking into account that BK induces bronchoconstriction and AHR in asthmatic patients [6], while BK antagonists reduce them significantly [1], it could be hypothesized that cathepsin K may exert a positive effect during asthma by inactivating proinflammatory kinins. We examined bronchial smooth muscles of chronically hypoxic rats [that is, 3 weeks in hypoxia; $pO_2 = 75$ mmHg (1 mmHg $\approx$ 133.3 Pa)], in which it was demonstrated that hypoxia, a condition often associated with chronic obstructive pulmonary disease, increased airway sensitivity [40,41]. As observed with normoxic rats, BK induced a contraction that was specifically impaired by a B2-type receptor antagonist (Hoe140, 1 μM). In the presence of cathepsin K, but not cathepsins B, L or E-64-inactivated cathepsin K, contraction was rapidly impaired (results not shown), confirming that blockade of the contractile response depends on the kininase activity of cathepsin K. It was noteworthy that cathepsin K prevented contraction of smooth muscles, despite hyper-responsiveness of hypoxic rats.

In summary, the presence of an unidentified kininase from macrophages has been reported and the involvement of new enzymes in BK catabolism was proposed [22,25]. Moreover, it has

been shown that thiol-dependent cathepsins are released from macrophages during lung inflammation [9], and their active forms, including cathepsin K, from human inflammatory BAL fluids have been characterized (C. Serveau-Avesque, M. Ferrer-Di Martino and G. Lalmanach, unpublished work). Taken together with the potency of cathepsin K to impair BK-dependent contraction of smooth-muscle cells from normoxic and hypoxic rats, the present results support the notion that cathepsin K fulfils the criteria to be a kininase of physiological interest. The ability of cathepsin K to degrade pro-inflammatory kinins, and subsequently to impair activation of BK receptors, may attenuate bronchoconstriction during airway obstructive diseases.

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