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Intravascular and interstitial degradation of bradykinin in isolated perfused rat heart

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1 Bradykinin (BK) has been shown to exert cardioprotective effects which are potentiated by inhibitors of angiotensin I-converting enzyme (ACE). In order to clarify the significance of ACE within the whole spectrum of myocardial kininases we investigated BK degradation in the isolated rat heart.

2 Tritiated BK (^{3}H-BK) or unlabelled BK was either repeatedly perfused through the heart, or applied as an intracoronary bolus allowing determination of its elution kinetics. BK metabolites were analysed by HPLC. Kininases were identified by ramiprilat, phosphoramidon, diprotin A and 2-mercaptoethanol or apstatin as specific inhibitors of ACE, neutral endopeptidase 24.11 (NEP), dipeptidylaminopeptidase IV and aminopeptidase P (APP), respectively.

3 In sequential perfusion passages, ^{3}H-BK concentrations in the perfusate decreased by 39% during each passage. Ramiprilat reduced the rate of ^{3}H-BK breakdown by 54% and nearly abolished [1-5]-BK generation. The ramiprilat-resistant kininase activity was for the most part inhibited by the selective APP inhibitor apstatin (IC_{50} 0.9 μM). BK cleavage by APP yielded the intermediate product [2-9]-BK, which was rapidly metabolized to [4-9]-BK by dipeptidylaminopeptidase IV.

4 After bolus injection of ^{3}H-BK, 10% of the applied radioactivity were protractedly eluted, indicating the distribution of this fraction into the myocardial interstitium. In samples of such interstitial perfusate fractions, ^{3}H-BK was extensively (by 92%) degraded, essentially by ACE and APP. The ramiprilat- and mercaptoethanol-resistant fraction of interstitial kininase activity amounted to 14%, about half of which could be attributed to NEP. Only the product of NEP, [1-7]-BK, was continuously generated during the presence of ^{3}H-BK in the interstitium.

5 ACE and APP are located at the endothelium and represent the predominant kininases of rat myocardium. Both enzymes form a metabolic barrier for the extravasated fraction of BK. Thus, only interstitial, but not intravascular concentrations of BK are increased by kininase inhibitors to the extent that a significant potentiation of BK effects could be explained. NEP contributes less than 5% to the total kininase activity, but is the only enzyme which is exclusively present in the interstitial space.

Keywords: Bradykinin; kininases; kinin metabolism; heart; angiotensin I converting enzyme; aminopeptidase P; neutral endopeptidase; apstatin

Introduction

Kinins are small peptide hormones which potently induce vasodilation through stimulation of vasodilator release from the endothelium (reviewed by Bhoola *et al*., 1992). This effect is mediated by endothelial kinin receptors of the B_2 subtype which are activated by agonists such as bradykinin (BK) or kallidin (Lys-BK). Endothelial mediators released by BK include prostacyclin (PG I_2), nitric oxide (NO) and as yet unidentified endothelium-derived hyperpolarizing factors, depending on the size and origin of the blood vessels investigated (Mombouli & Vanhoutte, 1992; Mombouli *et al*., 1992). In this respect kinins activate physiological mechanisms within the endothelium which may be protective, in the case of the ischemic heart, not only by improving perfusion but also by attenuating catecholamine release, inhibiting platelet aggregation and detoxifying oxygen radicals (Radomski *et al*., 1987; Schwieler *et al*., 1993; Minshall *et al*., 1994; Massoudy *et al*., 1995).

Kinins *in vivo* are rapidly degraded by various kininases and therefore, at least in the physiological state, are thought to act exclusively as local tissue hormones. Accordingly, it is the activity of local tissue kininases which primarily determines the effectiveness of kinins. These considerations also apply to the myocardium, as kinins can be formed in the heart (Baumgarten *et al*., 1993) by mechanisms which may involve the activity of locally expressed tissue kallikrein (Nolly *et al*., 1994).

Particular interest in the significance of cardiovascular kinin metabolism arose from the discovery that angiotensin I converting enzyme (ACE, E.C. 3.4.15.1) is an important kinin degrading enzyme, which implies that inhibitors of ACE have the potential to enhance the effects of kinins by increasing their half-life and availability. Since then, significant contributions of kinins to the various effects of ACE inhibitor therapy have repeatedly been demonstrated (reviewed by Linz *et al*., 1995).

There are, however, only a few studies in which enhancements of BK effects by ACE inhibition have been quantitated. Data obtained with anaesthetized rats or human volunteers revealed that the doses of intravenously applied BK required to induce comparable hypotensive effects were lowered 20 to 120-fold by ACE inhibition (Bönner *et al*., 1990; Ryan *et al*., 1994). These results seem to indicate a decisive role of ACE in kinin breakdown. However, comparative data on alternative degradation pathways in myocardium are not available. Neutral endopeptidase (NEP, E.C. 3.4.24.11) is the only kininase whose additional presence in the myocardium has been demonstrated (Piedimonte *et al*., 1994), whereas other kininases like aminopeptidase P (APP, E.C. 3.4.11.9), metalloendopeptidase (E.C. 3.4.24.15), post-proline-cleaving enzyme (E.C. 3.4.21.26) or carboxypeptidase M (E.C. 3.4.24.-) have not been investigated.

Aside from enzymatic activity, the localization of tissue kininases will also influence their function. In view of the proposed local formation of kinins in various tissues, kininase activities present in the interstitial space in particular deserve attention, as enzymes in this location could inactivate locally produced kinins on their way to the endothelial receptors.

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The aim of the present study was to carry out a biochemical identification of the kininases present in isolated rat heart. This model was chosen because it has been frequently used for investigation of kinin-dependent cardioprotection. For these experiments, rat hearts were retrogradely perfused with unlabelled or tritiated bradykinin (BK or ^{3}H-BK), and the peptide metabolites contained in the venous effluent were analysed by HPLC. The proportions of the various kininases present were determined by use of specific enzyme inhibitors.

Differentiation of intravascular from interstitial kinin degradation pathways was achieved by analysis of the kinetics of metabolite elution after application of a ^{3}H-BK bolus.

Methods

Preparation of isolated perfused rat hearts

Male Wistar rats (300–400 g body weight, Charles-River, Sulzfeld, F.R.G.) were anaesthetized with pentobarbital (50 mg kg^{-1}, i.p.). Hearts were prepared while they were continuously flushed with cold NaCl (0.9%, 4°C). Aortae were cannulated *in situ* and perfusion was initiated immediately. Hearts were transferred to a flow-through thermostat and were perfused at constant flow (5 ml min^{-1}) with Earle's balanced salt solution (116 mM NaCl, 26.2 mM $NaHCO_3$, 5.4 mM KCl, 1 mM NaH_2PO_4, 1.8 mM $CaCl_2$, 0.81 mM $MgSO_4$, 5.6 mM glucose, pH 7.4) which was equilibrated with 95% O_2, 5% CO_2 and warmed to 37°C. After ligation of all Vv. cavae, the A. pulmonalis was cannulated with a short (30 mm) polyethylene tube to allow complete collection of the venous effluent. Experiments were started within 15 min of completion of these procedures.

Degradation of bradykinin during consecutive perfusion passages

The kinetics of BK degradation were established by passaging BK in a fixed volume of 25 ml perfusion buffer repeatedly through the hearts. Either a low concentration (0.1 nM) of tritiated BK (^{3}H-BK) or high concentrations (3 or 20 μM) of unlabelled BK were used for perfusion. During the perfusion phases the venous effluent was collected and four samples of the effluent were drawn during each passage. Immediately after completion of a perfusion phase the collected effluent was reused for the next passage. Such myocardial perfusion passages were repeated five times consecutively. Samples of venous effluent (300 μl) were stabilized by addition of trifluoroacetic acid (1% final concentration) containing unlabelled BK (3 μg) whenever the radioactive substrate was used. In experiments addressing the effectivity of various peptidase inhibitors, hearts were pretreated with the inhibitors for 3–5 min prior to the application of the substrate solution. The effects of various inhibitors on BK degradation were determined in the same heart preparation by adding them successively over the course of the experiment.

Kinetics of metabolite elution following an intracoronary bolus of bradykinin

In order to compare kinin degradation activities in different distribution compartments, ^{3}H-BK was applied to the hearts as a bolus and the venous effluent was sampled with high temporal resolution. For a period of 6 s, normal perfusion of hearts (prepared as described above) was interrupted and 0.5 ml of perfusion buffer containing 6.1 nM ^{3}H-BK and 4 μM FITC-dextran were injected directly into the aorta. Even while the substrates were being injected, the venous effluent was sampled at intervals of 3 s into preweighed tubes containing 25 μl trifluoroacetic acid (10%). The volume of each fraction was determined by weight. The washout kinetics of the intravascular marker FITC-dextran and of labelled kinin peptides were established, respectively, either by measuring fluorescence at 450/510 nm or ^{3}H-radioactivity in aliquots of each effluent fraction. FITC-fluorescence was measured in 10 μl aliquots neutralized with 200 μl of phosphate buffer (400 mM Na_2HPO_4, pH 8). For determination of total ^{3}H-radioactivity, 30 μl aliquots of each effluent fraction were mixed with 10 ml scintillation cocktail (Hydroluma, Baker, Deventer, The Netherlands) and counted (1219 Rackbeta, LKB, Munich, F.R.G.). Whenever peptidase inhibitors were used in the experiments their administration was started 5 min prior to ^{3}H-BK injections and was continued throughout the sampling period.

Peptide analysis

The spectrum of BK degradation products was determined as described previously (Dendorfer *et al*., 1997). In brief, peptides were separated on an ET 200 Nucleosil 5 C_{18} column (Macherey-Nagel, Düren, F.R.G.), and were either detected at 210 nm or collected in 1.7 ml fractions and quantitated according to their β-radioactivity. Perfusates which had been subjected to sequential myocardial passages were analysed for ^{3}H-BK, [1-8]-BK, [2-9]-BK, [1-7]-BK, and [1-5]-BK, while analyses of perfusate fractions collected after ^{3}H-BK bolus applications also included [1-3]-BK, [2-3]-BK, [1-2]-BK, and proline.

Substances

$NaHCO_3$-free salt mixtures of Earle's balanced salt solution and FITC-dextran (molecular weight 500 000 g mol^{-1}) were obtained from Sigma, Deisenhofen, F.R.G. Peptide standards BK, [1-8]-BK, [2-9]-BK, [1-7]-BK, [1-5]-BK, [1-3]-BK, [2-3]-BK, [1-2]-BK and proline were acquired from either Sigma (Deisenhofen, F.R.G.) or Bachem (Bubendorf, Switzerland). [^{3}H-Pro^{2,3}]-bradykinin (^{3}H-BK, specific activity 2.22 TBq mmol^{-1}) was obtained from Du Pont (Bad Homburg, F.R.G). The purity of ^{3}H-BK was 97±1% when used for sequential perfusion passages and 96.4±0.1% when used for bolus injections. The concentrations of ^{3}H-BK were 0.1 nM in perfusion studies and 6.1 nM in bolus injections. The different peptidases were assessed by using specific inhibitors: ACE (E.C. 3.4.15.1) was inhibited by ramiprilat (250 nM); neutral endopeptidase 24.11 (NEP, E.C. 3.4.24.11) was inhibited by phosphoramidon (1 μM); dipeptidylaminopeptidase IV (DPAP, E.C. 3.4.14.5) was blocked by diprotin A (Ile-Pro-Ile, 100 μM); aminopeptidase P (APP, E.C. 3.4.11.9) was inhibited either by apstatin (0.1–10 μM) or 2-mercaptoethanol (1 mM). Ramiprilat and apstatin were generously supplied by Hoechst Marion Roussel (Frankfurt, F.R.G.) while all other inhibitors were obtained from Sigma (Deisenhofen, F.R.G).

Calculations and statistics

Concentrations of unlabelled BK in the perfusates were measured by HPLC and UV-detection (210 nm), and were related to the amounts of BK in the substrate solutions. Tritiated peptides were quantitated according to the ^{3}H-activities present in the individual peptide-yielding HPLC fractions after correction for basal activity present in unlabelled samples. In the case of sequential perfusion passages, these values were related to the total radioactivity of the substrate solution which was determined after HPLC as the sum of all peptides analysed. In experiments using ^{3}H-BK bolus applications, the effluent fractions contained variable amounts of radioactivity so that each peptide was related to the total radioactivity of each individual sample as determined after HPLC.

In order to quantitate the kinetics of ^{3}H-BK degradation during sequential myocardial passages, a monoexponential decline ($BK(n) = BK_0 * e^{-\beta * n}$, n = number of myocardial passages) was fitted to the profile of BK degradation. In experiments involving ^{3}H-BK bolus injections, a relative decrease of ^{3}H-BK during the single myocardial passage was calculated from the changes of relative ^{3}H-BK concentrations in the effluent fractions as compared to the substrate solution. Values

of relative decrease were transformed into degradation rate constants β according to the relationship: relative decrease per passage $= 1 - e^{-\beta}$. The contributions of certain kininases to the total kininase activity were calculated from the reductions of the degradation rate constants (β) brought about by specific enzyme inhibitors in relation to the degradation rate constant under control conditions.

Two effluent fractions collected after bolus injections of ^{3}H-BK were analysed for the relative contributions of the various kininases: An early fraction, sampled during substrate injection (3 s), when eluting ^{3}H-BK had passed the myocardium exclusively in the intravascular space, and a late fraction, sampled at 36 s, which represented BK that had been distributed into a larger compartment, presumably the interstitial space.

All quantitative data are given as means $\pm$ s.e.mean of five independent experiments. Inhibitory properties of apstatin were derived from dose-response curves fitted to each experiment. Parameters of ^{3}H-BK degradation were compared among the treatment groups by a paired *t*-test (sequential myocardial passages) or by analysis of variance with Tukey's post-hoc test (bolus injections). A time-dependency of kinin concentrations eluting from the interstitium after ^{3}H-BK bolus injections was tested by searching for differences within all samples obtained later than 30 s using analysis of variance for repeated measures. Differences were considered as being statistically significant at an error level of $P < 0.05$.

Results

Degradation of ^{3}H-BK during sequential passages through the heart

In experiments where sequential perfusion passages were enacted, concentrations of ^{3}H-BK and its metabolites in the cardiac effluent reached a steady state during the first minute after initiation of an individual passage and remained constant thereafter. For this reason only the last aliquot of each perfusion passage was analysed. ^{3}H-BK administered with the perfusate was degraded by $39 \pm 3\%$ per passage. The fragments [1-7]-BK, [1-5]-BK and, in small amounts, [2-9]-BK were the metabolites detected (Figure 1a). The levels of [1-8]-BK did not exceed 2.2%. After five perfusion passages only 49% of the radioactivity initially applied was recovered as the sum of all kinin fragments analysed, thus indicating a rapid further degradation. Small peptides like [1-3]-BK, [1-2]-BK, Pro-Pro or single proline were not investigated in these analyses.

In hearts pretreated with ramiprilat (250 nM) for 5 min, generation of [1-7]-BK was markedly reduced and that of [1-5]-BK was almost abolished, while formation of [2-9]-BK was slightly increased (Figure 1b). The relative degradation of ^{3}H-BK was $20 \pm 1\%$ per passage, which reflected a reduction of the BK degradation rate by 54% in comparison to control ($P < 0.05$). Production of [2-9]-BK indicated aminoterminal cleavage as a metabolic pathway in addition to ACE. Aminoterminal degradation, however, could not be further investigated in these experiments as the tritiated proline residues located at positions 2 and 3 of ^{3}H-BK were lost in the early course of degradation.

Metabolites of BK (20 μM) generated in the presence of various peptidase inhibitors

The pathways of aminoterminal degradation of bradykinin and the specificity of certain kininase inhibitors were investigated using unlabelled bradykinin (BK) at a high concentration (20 μM) which allowed detection of the kinins by UV-absorption at 210 nm (Figure 2a). During sequential passages through the heart, BK at this concentration was degraded at a considerably lower rate than ^{3}H-BK (relative degradation 24% per passage) and ramiprilat was slightly less effective in inhibiting BK breakdown (reduction of the degradation rate by

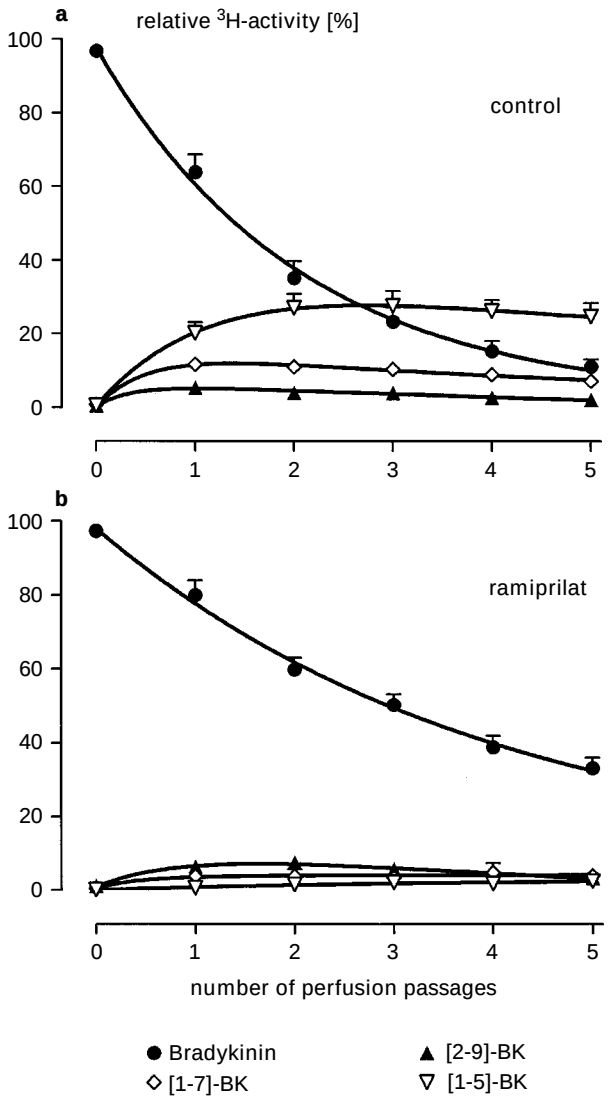

Figure 1 Kinetics of ^{3}H-bradykinin (^{3}H-BK) breakdown during sequential passages through isolated rat heart. Time courses of the relative amounts of ^{3}H-BK and its degradation fragments [1-7]-BK, [1-5]-BK, and [2-9]-BK in the venous effluent are depicted. The experiments were either performed without peptidase inhibitors (a) or after 5 min preincubation with and in the presence of ramiprilat (0.25 μM) (b). The relative degradation of ^{3}H-BK was reduced from 39 to 20% per passage by ramiprilat ($P < 0.05$). Data are means of five experiments; vertical lines show s.e.mean.

30%, data not shown). The spectrum of BK metabolites after five perfusion passages under control conditions confirmed the formation of [1-5]-, [1-7]- and [2-9]-BK, but [4-9]-BK could also be identified as an additional degradation product (Figure 2c). Similar to the experiments with ^{3}H-BK, ramiprilat completely abolished [1-5]-BK formation and substantially reduced the levels of [1-7]-BK. The residual generation of [1-7]-BK could be eradicated by additional application of phosphoramidon which inhibited the enzyme NEP (Figure 2e). Neither ramiprilat nor phosphoramidon significantly altered the appearance of [2-9]- or [4-9]-BK. The origin of [4-9]-BK as a secondary degradation product of [2-9]-BK was demonstrated by inhibition of DPAP using diprotin A which produced a substantial reduction of [4-9]-BK while the precursor [2-9]-BK accumulated (Figure 2f). The role of APP-activity in the preceding production of [2-9]-BK could be shown by use of 2-mercaptoethanol which is a rather selective inhibitor of APP at a concentration of 1 mM (Orawski *et al*., 1989) (Figure 2g). No significant BK-degrading activity was observed when ramiprilat, phosphoramidon and 2-mercaptoethanol were applied

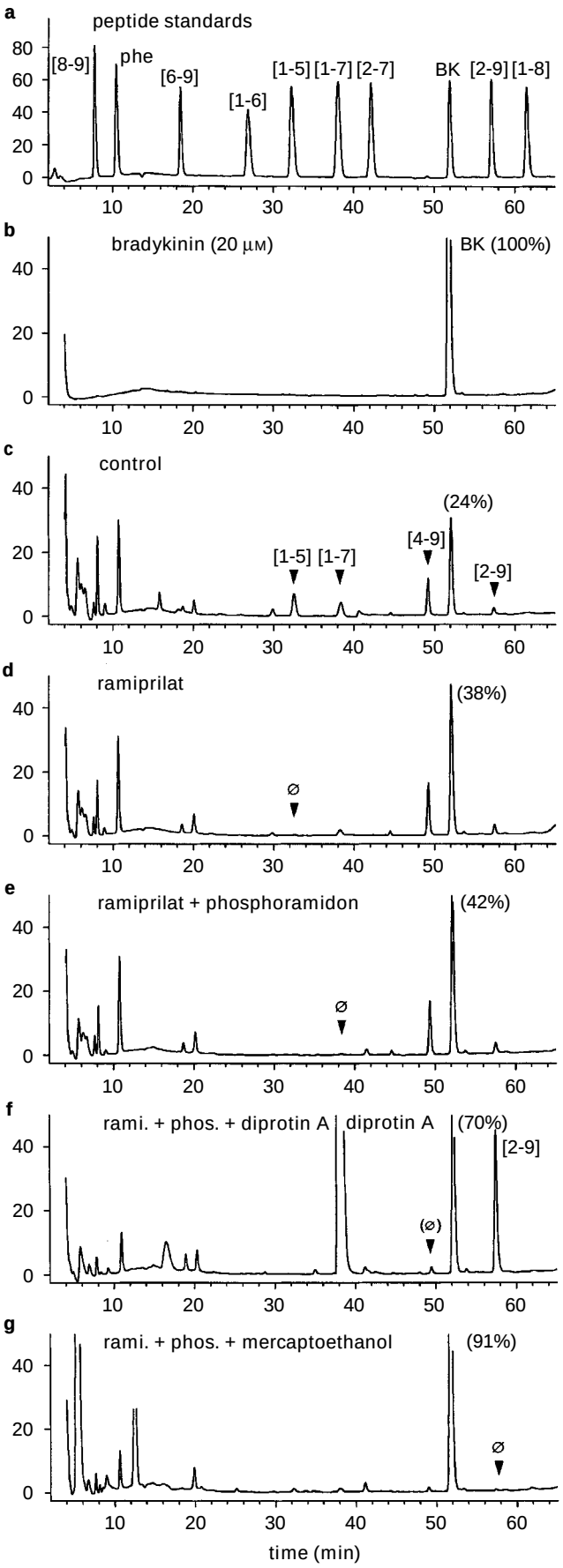

Figure 2 Identification of bradykinin metabolites in the perfusion medium after five coronary passages. Original HPLC tracings of standard peptides (a) or samples of the venous effluents from a typical experiment are depicted. Unlabelled bradykinin (BK, 20 μM) in 25 ml perfusion buffer was used as substrate (b). The metabolite spectra were determined in the perfusate after five consecutive passages through the coronary system performed either without peptidase inhibitors (c) or after pretreatment (5 min) with and in the presence of ramiprilat (0.25 μM), phosphoramidon (1 μM), diprotin A (Ile-Pro-Ile, 100 μM), and 2-mercaptoethanol (1 mM) in the combinations indicated. Ramiprilat alone abolished the generation of [1-5]-BK (d), but suppressed [1-7]-BK only in combination with phosphoramidon (e) which indicated the presence of angiotensin I-converting enzyme and neutral endopeptidase. Inhibition of dipeptidylaminopeptidase IV by diprotin A reduced the generation of [4-9]-BK and led to an accumulation of the intermediate product [2-9]-BK (f). The involvement of aminopeptidase P in the production of [2-9]-BK was demonstrated by the inhibitory effect of mercaptoethanol (g).

simultaneously (relative degradation 2% per passage), indicating that no major kininases were neglected in these analyses.

Inhibition of ramiprilat-resistant BK breakdown by apstatin

In order to substantiate the identification of the ramiprilat-resistant kininase activity as APP, a recently characterized selective inhibitor of APP, apstatin, was investigated in experiments employing sequential perfusion passages with 3 μM unlabelled BK (Prechel *et al*., 1995). Under these conditions, the basal rate of kinin degradation (37 ± 2% per passage) and the inhibitory effect of ramiprilat (54% inhibition) were identical to those observed with low concentrations of ^{3}H-BK. Application of apstatin in addition to ramiprilat led to a dose-dependent supplementary inhibition which at its maximum corresponded to 35 ± 2.7% of the total BK degradation rate (Figure 3). The IC_{50} of apstatin, as derived from the dose-effect curve, was 0.9 ± 0.16 μM.

Analysis of ^{3}H-BK metabolites eluting from different distribution compartments

In order to differentiate intravascular and interstitial kinin degradation, ^{3}H-BK (6.1 nM) in combination with the intravascular marker FITC-dextran (500 000 g mol^{-1}, 4 μM) was injected as a bolus directly into the aortic canula. Starting with the injection, the venous effluent was collected over 60 s

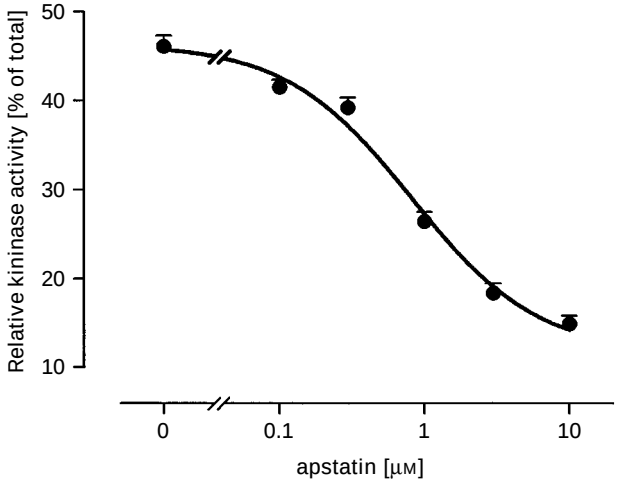

Figure 3 Inhibitory effect of apstatin on ramiprilat-resistant myocardial kininase activity. Degradation kinetics were assessed with five sequential perfusion passages of unlabelled bradykinin (BK, 3 μM). The rate of BK degradation was reduced to 46% of its control value by application of ramiprilat (0.25 μM). Additional application of apstatin dose-dependently inhibited the residual kinin breakdown. Fitting of a dose–response curve indicated that 35% of the total myocardial kininase activity could be inhibited by apstatin with an IC_{50} concentration of 0.9 μM. Data are means of five experiments; vertical lines show s.e.mean.

at 3 s intervals. In all these samples, total recovery of ^{3}H-BK was 80±4% and that of FITC-dextran 83±2%. At the end of substrate injection, FITC-dextran in the venous effluent decreased with a rapid, monophasic time course (Figure 4). ^{3}H-radioactivity corresponding to ^{3}H-BK and its metabolites

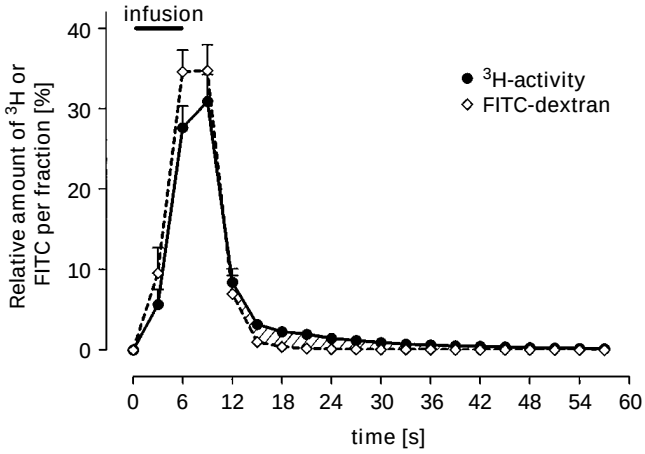

Figure 4 Elution kinetics of ^{3}H-radioactivity and FITC-fluorescence after combined application of ^{3}H-bradykinin (^{3}H-BK) and FITC-dextran as an intracoronary bolus. Venous effluent from isolated hearts was collected at intervals of 3 s, starting with an injection of ^{3}H-BK (3.05 pmol) and FITC-dextran (500 000 g mol^{-1}, 2 nmol) over 6 s. The absolute contents of each effluent fraction was related to the total doses applied. In contrast to the intravascular marker FITC-dextran a 10% fraction of ^{3}H-radioactivity was slowly eluted from the heart (hatched area), indicating its distribution into a larger compartment which presumably corresponded to the interstitial space. Data are means of five experiments; vertical lines show s.e.mean.

was eluted from the heart in a biphasic manner. This indicated the existence of an additional distribution compartment, presumably the interstitial space (Figure 4). The difference of the areas under the concentration curves of ^{3}H-radioactivity and FITC-dextran in the late phase of elution (12 s and more, hatched area in Figure 4) amounted to 10±0.3% of the applied dose of ^{3}H-BK under control conditions. When the decline of ^{3}H-radioactivity efflux (sampling time 9 s and more) was fitted to a biexponential time course, a half-life of 11.4 s was calculated for the slow phase of ^{3}H-washout. Assuming that this delayed elution was due to a redistribution of ^{3}H-labelled peptides from an interstitial compartment which exchanged substances with the perfusate by virtue of a steady flow of interstitial fluid, the volume of this interstitial distribution compartment can be calculated. According to this model, the interstitial flow rate should correspond to the extravasated fraction of the perfusion rate (10% extravasated ^{3}H-activity/80% total ^{3}H-recovery *5 ml min^{-1} perfusion rate = 0.625 ml min^{-1} interstitial flow). When this flow rate is able to induce a fractional ^{3}H-efflux rate of 0.061 s^{-1} (ln 2/11.4 s), the apparent distribution volume of the interstitial compartment is represented by the ratio of interstitial flow and fractional efflux rate. The calculated value of 171 μl is within the range expected for the interstitial space of 1 g of myocardium under physiological conditions, thereby confirming that infused kinins have access to and become distributed within the interstitial space as a whole.

The kinetics of kinin elution after bolus injections revealed that only a minor fraction of ^{3}H-BK was degraded when the peptide had remained in the intravascular space during its myocardial passage (72±1.7% intact ^{3}H-BK at 3 s) (Figure 5a). However, ^{3}H-BK degradation became more and more extensive after the substrate injection was terminated (7.8±1% intact ^{3}H-BK at 36 s) when kinins which were redistributed from the interstitial space contributed increasingly to the kinin

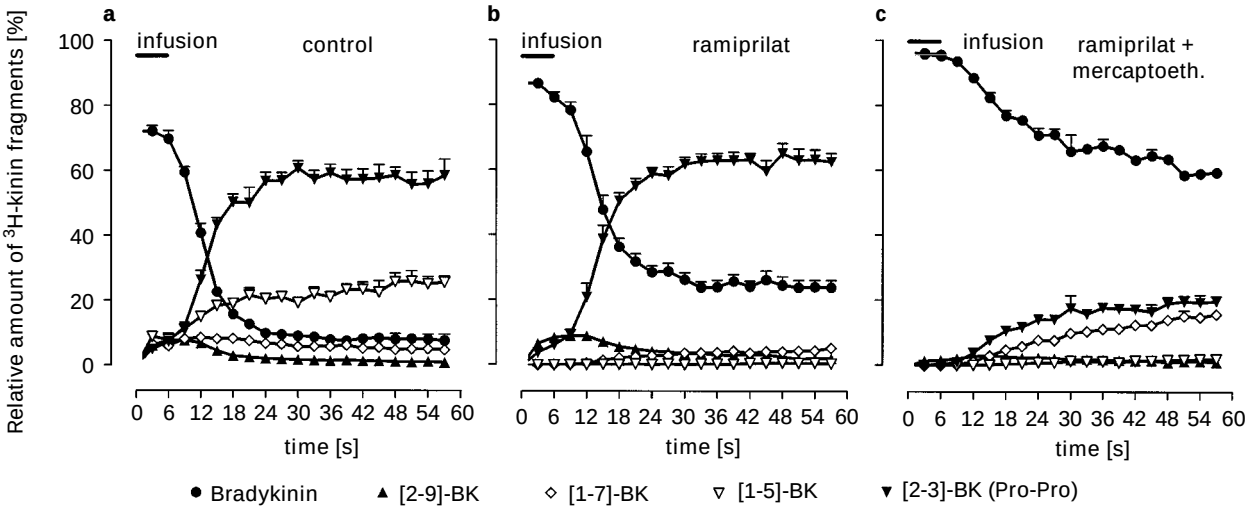

Figure 5 Elution kinetics of kinin metabolites after intracoronary bolus application of ^{3}H-bradykinin (^{3}H-BK). The relative amounts of ^{3}H-BK and its metabolites [1-7]-BK, [1-5]-BK, [2-9]-BK, and [2-3]-BK (Pro-Pro) in each fraction of the venous effluent are depicted. The experiments were performed without kininase inhibitors (a), and in the presence of ramiprilat (0.25 μM) and 2-mercaptoethanol (1 mM) for inhibition of angiotensin I-converting enzyme (ACE) alone (b) or in combination (c) with aminopeptidase P (APP). Production of [1-7]-BK and [1-5]-BK under control conditions reflected the combined activities of ACE and neutral endopeptidase (NEP). The fragments [2-9]-BK and [2-3]-BK (Pro-Pro) were generated by the sequential actions of dipeptidylaminopeptidase IV and APP. In early effluent fractions (3–6 s), which represented the intravascular breakdown of ^{3}H-BK, only a minor portion of ^{3}H-BK was degraded. In these fractions, the generation of [1-7]-BK and [1-5]-BK was abolished by ramiprilat, indicating the presence of ACE and the absence of NEP at the luminal endothelial surface. Additional inhibition of APP by mercaptoethanol largely reduced the formation of [2-9]- and [2-3]-BK. At later elution times (30–60 s), when kinins were redistributed from the interstitium, ^{3}H-BK was extensively degraded, again with the essential involvement of ACE and APP. The kininase activities resistant to ramiprilat and mercaptoethanol led to the formation of [1-7]-BK and [2-3]-BK, and could therefore be attributed to the activity of NEP and an incomplete inhibition of APP. Only when the major kininases, ACE and APP were inhibited, a time-dependent decrease of ^{3}H-BK during the second half of the experiment was observed ($P<0.05$). This was paralleled by a significant time-dependent increase of [1-7]-BK ($P<0.05$), but not of [2-3]-BK, indicating the continuous activity of NEP in the interstitial compartment. Data are means of five experiments; vertical lines show s.e.mean.

contents of the effluent. The spectrum of kinin metabolites included [1-5]-, [1-7]- and [2-9]-BK which also had been identified after consecutive perfusion passages as well as [2-3]-BK (Pro-Pro) which is the labelled product of the degradation of [2-9]-BK by DPAP (Figure 5a). The second-step generation of [2-3]-BK is reflected by the enhanced formation of the secondary product [2-3]-BK at the expense of [2-9]-BK under the conditions of more extensive metabolism during the late phase of kinin elution (Figure 5a). Further possible products or intermediates of ^{3}H-BK degradation were detected only in very small amounts ([1-8]-, [1-3]-, [1-2]-BK less than 1.5%; proline less than 3.5%). Whereas a clear difference in kinin metabolism between an early and a late phase of elution was evident, no further time-dependent changes in the spectrum of kinin metabolites occured in the second half of the sampling period (Figure 5a). It can be concluded that ^{3}H-BK was rather stable once it had reached the larger distribution compartment, so that the major part of degradation must have occured earlier.

With the use of ramiprilat, formation of [1-5]-BK was abolished and ^{3}H-BK was better preserved in the early ($87\pm0.9\%$ intact ^{3}H-BK at 3 s, $P<0.05$) as well as in the late phase ($24\pm2\%$ intact ^{3}H-BK at 36 s, $P<0.05$) of elution (Figure 5b). The relative concentrations of [2-3]-BK were little affected by ramiprilat. The early and rapid formation of [1-7]-BK was absent, but a continuous increase was now observed in the course of the experiment (Figure 5b).

Administration of mercaptoethanol in addition to ramiprilat substantially reduced, but did not abolish the aminoterminal degradation products [2-9]-BK and Pro-Pro, indicating that the aminopeptidase P-like activity was not completely inhibited under these conditions (Figure 5c). Compared to ramiprilat alone, preservation of ^{3}H-BK was improved during the whole time-course ($96\pm1\%$ intact ^{3}H-BK at 3 s, $68\pm2\%$ at 36 s, $P<0.05$) and the formation of [1-7]-BK was increased. Under these conditions a significant time-dependent reduction in ^{3}H-BK occured over the period between 30 and 60 s ($P<0.05$). This decrease was paralleled by an increase of [1-7]-BK ($P<0.05$) while the contribution of [2-3]-BK remained constant (Figure 5c).

When the relative decreases of degradation rate constants brought about by each of the kininase inhibitors were used to describe the relative contributions of the various enzymes towards kinin degradation, the distribution of myocardial kininases in the two distribution compartments of ^{3}H-BK could be determined, which will further be addressed as 'intravascular' or 'interstitial' activities. By this reckoning interstitial kininase activity was found to be 8.7 times higher than the intravascular activity. The activities of ACE and APP represented the major kininase activities in both compartments (Figure 6). While the contribution of ACE was slightly lower in the interstitial as compared to the intravascular space, a striking asymmetry of distribution was only seen for the fraction of kininase activity resistant to ramiprilat and mercaptoethanol. This activity played only a minor role in intravascular ^{3}H-BK degradation ($1.1\pm0.4\%$ of total kininase activity in the 3 s elution fraction), but contributed significantly to interstitial degradation pathways ($13.9\pm1.1\%$ of total kininase activity in the 36 s elution fraction) (Figure 6). The ramiprilat- and mercaptoethanol-resistant kininase activity could be delineated by the spectrum of kinin metabolites produced. While generation of [2-9]-BK and Pro-Pro could be explained by a residual activity of aminoterminal ^{3}H-BK cleavage, the metabolite [1-7]-BK which arose exclusively in the interstitial fraction (Figure 5) must have been produced by an additional mechanism. The enzyme responsible for this reaction could be identified as NEP according to the inhibitory effect of phosphoramidon on the ramiprilat-resistant generation of [1-7]-BK in the perfusion studies using 20 μM BK (Figure 2). The contribution of NEP to the ramiprilat- and mercaptoethanol-resistant kininase activity in the interstitial elution fraction after injection of a BK bolus could be estimated as approximately 40% according to the ratio of carboxy- to aminoterminal cleavage products of ^{3}H-BK (Figure 5c).

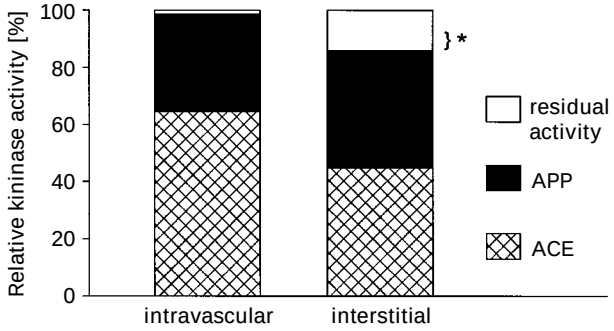

Figure 6 Contribution of angiotensin I-converting enzyme (ACE) and aminopeptidase P (APP) to the total kininase activities present in the intravascular and the interstitial space. Kininase activities were assessed by the inhibitory effects of ramiprilat (0.25 μM) and 2-mercaptoethanol (1 mM), respectively, on ^{3}H-bradykinin (^{3}H-BK) degradation in an intravascular (3 s) and an interstitial (36 s) effluent fraction after application of ^{3}H-BK as an intracoronary bolus. ACE and APP were the most important kininase activities in both compartments. The contributions of ACE, and of the kininase activity resistant to ramiprilat and mercaptoethanol, showed significant differences between the interstitial and the intravascular space ($P<0.05$). According to the generation of [1-7]-BK in the presence of ramiprilat and mercaptoethanol, about half of the inhibitor-resistant (residual) BK degradation activity in the interstitium can be attributed to the activity of neutral endopeptidase, as indicated in the graph (*). Data are means of five experiments.

Discussion

Identification and localization of rat myocardial kininases

From this study it has become clear that ACE is not the only relevant kininase present in rat myocardium. The role of this enzyme has been emphasized in numerous studies over the past decade. The special attention paid toward ACE is due to its ubiquitous presence in blood vessels throughout the body, as well as to the availability, clinical impact and kinin potentiating efficacy of ACE inhibitors. This study agrees with the characterization of myocardial ACE as a vascular kininase (Yamada *et al*., 1991) and provides information on the significance of this enzyme for BK breakdown in the heart. There are no previous data comparing myocardial BK degradation pathways. Such informations exist for BK degradation in rat plasma and in the rat pulmonary circulation where contributions of 47% and 50% for ACE within total kininase activities have been reported respectively (Ishida *et al*., 1989; Pesquero *et al*., 1992). Hence, there is no indication that ACE should play a more extensive role in BK degradation in other rat tissues.

The activity of APP is responsible for most of the remaining metabolism of BK. The identification of APP can solely be based on the demonstration of its direct cleavage product, [2-9]-BK, as it is the only peptidase known to remove arginin from the aminoterminal end of BK. This conclusion is confirmed by the inhibitory actions of mercaptoethanol and apstatin (Orawski *et al*., 1989; Prechel *et al*., 1995). Apstatin in particular has been described as a selective inhibitor of APP. It even exerted a slighly higher potency in the intact heart (IC_{50} 0.9 μM) compared to purified APP (K_i 2.6 μM, according to Prechel *et al*., 1995). The observation of a potent inhibition of APP by mercaptoethanol (Figure 2) which led to the same reduction of myocardial kininase activity as apstatin (Figures 3 and 6) justifies the use of mercaptoethanol for inhibition of APP, as has been suggested earlier (Orawski *et al*., 1989).

APP has not been investigated as extensively as ACE. It has been detected in soluble form in rat serum, and contributes as a membrane-bound enzyme to the degradation of BK in the rat lung (Ward *et al*., 1991; Pesquero *et al*., 1992; Ryan *et al*., 1994). The distribution of APP has been established in various human organs where higher activities were located on platelets

as compared to serum (Scharpe *et al*., 1991; Vanhoof *et al*., 1992). Myocardium, however, was not included in any of these studies. Therefore, this is the first demonstration of APP in rat myocardium, and the first identification of myocardial APP as an endothelial enzyme. It should be stated, however, that this and all previous quantitative investigations on APP are based only upon measurements of catalytic activity. Demonstration of APP by immunocytochemistry or expression analysis has until now only been achieved in one study on cultured human umbilical vein endothelial cells (Ryan *et al*., 1996)

The cellular localizations of ACE and APP in rat heart appear to be quite similar as both contribute by about the same proportions to the degradation of intravascular as well as interstitial BK. Clearly, both enzymes are present at the luminal surface of the endothelium because their activities determine the metabolite spectrum in the intravascular elution fraction of a BK bolus. In addition, both kininases essentially contribute to the nearly complete degradation of ^{3}H-BK in the interstitial elution fractions. However, under control conditions, no time-dependent progression of kinin metabolism in the interstitium was observed. This indicates that probably both ACE and APP do not continuously degrade BK during its presence in the interstitium, but rather operate in a one-step process acting while filtration of the substrate through the vessel wall occurs. This kinetics would be consistent with an exclusively endothelial localization and possibly an additional presence of both enzymes at other vascular cells types such as smooth muscle cells or pericytes. However, these results obtained in healthy hearts do not indicate an expression of ACE in cardiac myocytes or fibroblasts as has been observed in cultured cells (Dostal *et al*., 1992), and which may in fact occur *in vivo* during the development of cardiac hypertrophy and remodelling (Schunkert *et al*., 1990; Hirsch *et al*., 1991).

NEP, which is the third kind of kininase found in rat myocardium, is responsible for the selective formation of [1-7]-BK in the presence of ramiprilat and mercaptoethanol. The identity of the enzyme was proven by the inhibitory effect of phosphoramidon on [1-7]-BK generation in perfusion studies using unlabelled BK. The quantitative significance of the enzyme is low. It does not take part in the degradation of intravascular kinins and contributes less than 10% to interstitial kininase activities. However, its exclusively interstitial localization is a unique feature of this kininase, which is the only enzyme responsible for the continuous degradation of ^{3}H-BK in the interstitium. The cellular localization of NEP can only be speculated upon. The absence of NEP at the intravascular surface of the endothelium reported here appears to contrast with the demonstration of NEP on cultured endothelial cells (Llorens-Cortes *et al*., 1992; Graf *et al*., 1995). An exclusive localization of the enzyme at the basal membrane of endothelial cells, however, would still be compatible with both findings. More plausible sources of interstitial NEP, however, may either be the myocytes, or tissue leukocytes (Connelly *et al*., 1985; Piedimonte *et al*., 1994). The functional significance of NEP should not be underestimated simply because it has relatively low activity against the exogenous kinins reported here. In experiments where endogenous BK was released in the myocardium by stimulation of sensory neurons, inhibition of NEP provoked a significant potentiation of kinin-mediated vasodilation (Piedimonte *et al*., 1994).

An enzyme involved in processing the metabolite [2-9]-BK further was identified as DPAP in this study and its activity in rat myocardium was found to exceed the activity of APP by far. This result agrees with the histochemical localization of DPAP in capillaries of rat skeletal muscle (Mrázková *et al*., 1986). As DPAP did not contribute toward inactivation of BK itself, its distribution and activity were not further characterized.

It should be pointed out that the quantitation of BK breakdown as relative degradation per passage reflects the functional significance, but not absolute activities of the various kininases. The extensive degradation of the extravasated ^{3}H-BK can be brought about by a minor fraction of myocardial kininase activity when this can act very effectively due to the low exchange rate of interstitial fluid and the close contact of BK to vascular kininases during extravasation. The three identified kininases presumably vary in substrate affinity: an additional factor which can influence BK degradation activity. ACE and APP have similar and strong affinities for BK (K_m or binding affinities of 0.9 – 17 μM and 1.1 – 19.7 μM, respectively, according to Dorer *et al*., 1974; Drapeau *et al*., 1991; Ward *et al*., 1991; Ryan et al., 1992), whereas NEP has a lower affinity (K_m of 120 μM, according to Gafford *et al*., 1983). Hence the minor role of NEP observed here could be a result of this low affinity rather than low expression.

Taken together the kininase activity distributions presented here may not be compatible with histochemical findings, but they should reflect the significances of the individual kininases under physiological conditions. Furthermore, by conducting experiments with low concentrations of BK, we have provided information which should be representative for all conditions where BK concentrations are lower than the K_ms of the individual kininases. This should include all physiological and pathophysiological situations *in vivo*.

Considerations on the potentiation of BK effects by kininase inhibitors

This study suggests a similar functional distribution and quantitative significance of the two predominant kininases in rat myocardium, ACE and APP. This finding justifies new discussions on the physiological role of kinin degradation and its modulation by kininase inhibitors. The total intravascular kininase activity degraded only 28% of the intravascular BK during a single myocardial passage when plasma kininases were absent. Under these conditions even complete kininase inhibition can bring about only a small increase of intravascular kinin levels. Due to the higher extent of kinin degradation in the interstitial space, a greater effect of kininase inhibition would be predicted for this compartment. The concentrations of intact BK in the interstitial space increased by factors of 3.1 and 8.7 after inhibition of ACE and ACE plus APP, respectively.

Previous results on the potentiation of BK effects by ACE inhibitors obtained in different models do not correspond to the small increase of intravascular kinin levels observed during ACE inhibition in this study. Depending on the model used, the potency of BK was increased 10- to 120-fold by ACE inhibitor treatment (Bönner *et al*., 1990; Mombouli & Vanhoutte, 1992; Minshall *et al*., 1994; Ryan *et al*., 1994). APP inhibitors appear to exert smaller kinin-potentiating effects as compared to ACE inhibitors. Potentiation by factors of 1.8 to 3.6, and about twofold stronger hypotensive effects of BK after intraarterial or intravenous applications have been achieved in rats through inhibition of APP (Ryan *et al*., 1994; Kitamura *et al*., 1995). However, even these effects would imply a more pronounced increase in kinin concentrations than could be induced by APP inhibition in the intravascular space of rat heart. Only kinin levels in the myocardial interstitium were found to be increased by kininase inhibition to the extent that they would correspond to a significant potentiation of kinin actions.

Several theories might explain a kinin-potentiating effect of ACE inhibitors higher than that expected from its effects on myocardial kinin metabolism. Under *in vivo* conditions, the activity of intravascular kinin metabolism will clearly be more significant due to the presence of circulating plasma kininases which consist mainly of ACE and carboxypeptidase N together with APP and other, unidentified enzymes (Ahmad & Ward, 1992; Ishida *et al*., 1989). Furthermore, non-selective inhibition of APP or other kininases may have occurred in experiments using high doses of ACE inhibitors (Hooper *et al*., 1992). Another interesting hypothesis implies that ACE inhibitors might induce kinin-mediated effects in a manner not related to their actions on kinin degradation. Such a mechanism would possibly involve an interaction with kinin re-

ceptors or kinin signal transduction (Auch-Schwelk *et al*., 1993, Hecker *et al*., 1994). Our results emphasize the importance of investigating this, especially as such direct kinin-mimetic effects of ACE inhibitors have not yet been reported in rat tissues. As a novel finding, the present study identified a metabolic barrier for kinins which is located between the intravascular and the interstitial space of the rat heart. In a situation where such a barrier would also limit BK concentrations at the site of kinin receptors, the inactivation of this barrier by kininase inhibitors would increase local kinin concentrations very effectively. This hypothesis would imply that most endothelial kinin receptors would not have direct contact with the intravascular space, as it is generally assumed.

In conclusion, this study identified ACE and APP as the predominant kininases of rat myocardium. These enzymes are located at the luminal surface of the endothelium and at other vascular sites which kinins pass by during extravasation. As such, ACE and APP form a metabolic barrier which effectively reduces kinin concentrations in the interstitium. A third kininase, NEP, makes up less than 5% of total kininase activity in the heart. This enzyme is not present at the intravascular surface, but is the only kininase which can continuously degrade BK in the interstitial space. The significance of NEP for tissue-derived peptides may therefore exceed its overall quantitative contribution. The findings of this study enable us to investigate kinin effects under conditions of more complete inhibition of kinin degradation and, perhaps, to utilize the concept of cardioprotection by kinin potentiation more effectively.

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