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Inhibition of kinin breakdown prolongs retention and action of bradykinin in a myocardial B_2 receptor compartment

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1 The high efficacy of ACE inhibitors to potentiate the actions of kinins might be explained by a hypothetical compartment in which B_2-receptors are colocalized with kinin degrading enzymes. To demonstrate the functional consequence of such a compartment we compared the myocardial uptake and the persistence of action of bradykinin under the influence of kininase inhibitors.

2 Bradykinin-induced vasodilation and uptake of tritiated bradykinin were studied in perfused rat hearts during inhibition of ACE and aminopeptidase P. B_2-receptors were localized by immuno-gold labelling and electron-microscopy.

3 The EC_{50} of bradykinin-induced vasodilation (5.1 ± 0.8 nM) was shifted to 14 fold lower concentrations during inhibition of both kininases. The maximum persistence of vasodilation after termination of bradykinin application (half-life 112 ± 20 s) was increased by kininase inhibitors to 398 ± 130 s. This prolongation was reversed when B_2-receptors were blocked simultaneously with the termination of bradykinin infusion.

4 Tritiated bradykinin (perfused for 1 min) was partially ($1.7 \pm 0.24\%$) retained by the myocardium and consecutively released with a half-life of 70 ± 9 s. Kinin uptake was increased during kininase inhibition ($7.7 \pm 2.6\%$), and was normalized by HOE 140 ($2.0 \pm 0.34\%$), or when a tritiated B_2-receptor antagonist (NPC 17731) was used as label.

5 B_2-receptors were localized in plasmalemmal and cytosolic vesicles of capillary endothelium.

6 Bradykinin is locally incorporated and can associate with B_2-receptors repeatedly when kinin breakdown is inhibited. This is the kinetic and functional consequence of a colocalization of kininases and B_2-receptors in a compartment constituted by endothelial membrane vesicles.

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Keywords: Bradykinin; angiotensin I-converting enzyme; aminopeptidase P; B_2 receptors; caveolae; rat; heart

Abbreviations: ACE, angiotensin I-converting enzyme; APP, aminopeptidase P; BK, bradykinin; ^{3}H-BK, [^{3}H-Pro^{2,3}]-bradykinin; TBS, TRIS-buffered saline

Introduction

A variety of beneficial actions of angiotensin I-converting enzyme (ACE) inhibitors have been attributed to their potentiation of the effects of endogenous kinins. Experimental studies have demonstrated that kinins are involved particularly in the attenuation of ischaemic damage, in the prevention of myocardial remodelling and fibrosis, and in inhibition of myocardial and vascular hypertrophy, effects that have all been observed with ACE inhibitor treatment (reviewed by Linz *et al*., 1995). However, the mechanisms by which ACE inhibitors provoke kinin potentiation have not yet been unequivocally clarified. Although inhibition of ACE-mediated kinin degradation clearly has the potential to enhance kinin actions, additional degradation-independent actions of ACE inhibitors have been postulated in order to explain the observed peculiarities of kinin potentiation completely. The requirement for such degradation-independent mechanisms arose from observations showing that ACE inhibitors (1) potentiate kinin actions to an extent far exceeding the detectable kinin accumulation (Auch-Schwelk *et al*., 1993; Hecker *et al*., 1997), (2) enhance the effects of slowly degraded kinin analogues (Auch-Schwelk *et al*., 1993; Minshall *et al*., 1997), (3) show structure-related differences in their kinin-potentiating abilities (Hecker *et al*., 1997) and (4) provoke a kinin-mediated response even when B_2-receptors have been desensitized by kinin pretreatment (Marcic *et al*., 2000; Minshall *et al*., 1997). A number of ACE inhibitor actions have been identified in different models that may constitute a degradation-independent basis for kinin potentiation. For example, ACE inhibitors can stabilize B_2-receptors in a high affinity state (Minshall *et al*., 1997), attenuate receptor sequestration and internalization (Benzing *et al*., 1999; Minshall *et al*., 1997), and enhance the spontaneous activity of unoccupied B_2-receptors (Hecker *et al*., 1997). However, the evidence for any of these mechanisms contributing to the functional potentiation of kinin effects under physiological conditions is lacking.

Recent studies on kinin-mediated vasodilation have confirmed the high extent of potentiation and the ability of ACE inhibitors to restore kinin actions after desensitization. However, such effects were abolished when ACE-resistant kinin analogues were used in place of bradykinin (BK) (Dendorfer *et al*., 2000; 2001; Gobeil *et al*., 2002; Tom *et al*.,

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2002). Since these observations exclude a degradation-independent action of the ACE inhibitor in different physiological models, a several-fold increase of BK availability at the functional B_2-receptors must be induced by ACE inhibitors in order to explain the observed magnitude of potentiation. In the rat heart, such substantial changes are not reflected in the BK concentrations of the perfusate (Dendorfer *et al*., 2000), and therefore it has been hypothesized that kinin accumulation must be restricted to a minute, B_2-receptor containing compartment. The existence of a myocardial distribution compartment enabling an 8.5 fold increase of BK concentrations after inhibition of kinin breakdown could indeed be verified using tracer transit studies (Dendorfer *et al*., 1997), but the identity of this compartment and its significance for B_2-receptor stimulation remained elusive.

The aim of the present study was to substantiate further the evidence for a colocalization of kinin degrading enzymes (kininases) with B_2-receptors in a functional myocardial subcompartment. The definition of this proposed compartment by a diffusion barrier and its endowment with a highly active local kinin metabolism present the possibility that degradation constitutes the principle mechanism of kinin elimination from this compartment. This hypothesis would predict that the retention of BK in the compartment will be prolonged when this major pathway of elimination is inhibited. As a consequence, the effects of BK will persist longer, not because of an alteration of receptor binding or signalling, but due to a repeated association of locally trapped BK with B_2-receptors within this compartment.

Methods

Experimental protocol

Isolated perfused Langendorff hearts were prepared from anaesthetized (pentobarbitone, 80 mg kg^{-1}, i.p.) male Wistar rats (250–300 g body weight, Charles-River, Sulzfeld, Germany) and were perfused at 15 ml min^{-1} with Krebs-Ringer solution as previously described (Dendorfer *et al*., 1997). Perfusion pressure was continuously recorded and served as a marker of coronary resistance. Drugs were applied as stock solutions (100 fold final concentrations) which were added to the perfusion medium immediately before the aortic canula. The major myocardial kininases, ACE and aminopeptidase P (APP), were inhibited by 250 nM ramiprilat and 1 mM mercaptoethanol, respectively (Dendorfer *et al*., 1997). Infusion of these inhibitors was started 10 min prior to the first application of BK. Dose/response curves of BK-induced vasodilation were obtained by 1 min applications of increasing BK doses (0.01–100 nM) which were given sequentially after stabilization of perfusion pressure. Myocardial uptake and release of kinins was determined by a 1 min application of 2 pmol of tritiated bradykinin (^{3}H-BK) or the tritiated B_2-receptor antagonist ^{3}H-NPC 17731 into the aortic canula. Starting with the injection, the coronary effluent was collected at 30 s intervals. After an additional 15 min of perfusion, the hearts were analysed for radioactivity. For extraction of labelled kinins, effluent fractions were dried under vacuum and dissolved in 1 ml trifluoroacetic acid (12 mM). Whole hearts were hydrolyzed in two parts (v w^{-1}) 6 M KOH (10 h, 95°C). After neutralization with perchloric acid, a clear supernatant was obtained by centrifugation ($5000 \times g$, 30 min). One ml of the myocardial extract or 1 ml of the concentrated perfusate were mixed with 10 ml scintillation cocktail (Hydroluma, Baker, Deventer, The Netherlands) and counted (1219 Rackbeta, LKB, Munich, Germany). Counting efficiency in the myocardial samples was determined by subsequent addition of ^{3}H-clonidine as an internal standard. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Localization of B_2-receptors

Myocardial samples were excised from isolated rat hearts after 20 min perfusion, and were fixed with 2% paraformaldehyde and 0.05% glutaraldehyde in phosphate-buffered saline, pH 7.4, for 1 h at 4°C. Following dehydration in a graded acetone series, the samples were embedded in LR White (London Resin Co., Reading, U.K.). Ultrathin sections were mounted on 300 mesh nickel grids and were blocked for 30 min with 0.5% bovine serum albumin (Sigma, Deisenhofen, FRG) in TRIS-buffered saline (TBS). The sections were incubated for 16 h with the primary antibody, diluted 1 : 1000 with TBS. After rinsing with TBS, sections were incubated for 2 h with a donkey-anti-rabbit IgG antibody coupled to 6 nm gold particles (Jackson Immuno Research, West Grove, U.S.A.), diluted 1 : 100 with TBS. Sections were contrasted with uranyl acetate and lead citrate and were examined with a Philips EM 400 electron microscope. In control incubations, a non-immune rabbit serum was substituted for the primary antibody.

Substances

Ramiprilat and HOE 140 (icatibant) were kindly donated by Prof G. Wiemer, Aventis Pharma AG, Frankfurt, Germany. [^{3}H-Pro^{2,3}]-bradykinin (^{3}H-BK, specific activity 3.3 TBq $mmol^{-1}$) and ^{3}H-NPC 17731 (D-Arg[Hyp^3, D-HypE(trans-propyl)^7, Oic^8]-bradykinin, specific activity 1.3 TBq $mmol^{-1}$), an antagonist with an identical affinity for the B_2-receptor (Herzig & Leeb-Lundberg, 1995; Nardone & Hogan, 1994), were obtained from Du Pont, Bad Homburg, Germany. Prior to the experiments, tritiated kinins were purified by HPLC as described elsewhere (Dendorfer *et al*., 1997). The B_2-receptor specific rabbit antiserum was kindly provided by Prof W. Müller-Esterl, Institute of Biochemistry II, University Frankfurt, Germany. Its specificity and suitability for histologic labelling has been reported elsewhere (Figueroa *et al*., 1995). All other chemicals were obtained at the highest grade available from Merck, Darmstadt, Germany or Sigma, Deisenhofen, Germany.

Calculations and statistics

BK-induced vasodilation was analysed for the maximum reduction in perfusion pressure during BK infusion, and for the kinetics of pressure readaptation after termination of BK infusion. The rate of pressure increase (k) was calculated by fitting a monoexponential function ($p(t) = p_{basal} - \Delta p * e^{-k*t}$) to

the complete time course of the perfusion pressures after BK infusion, and is expressed as half-life (ln2/k). Similarly, the release rates of tritiated peptides from the ^{3}H-BK labelled myocardium were derived from a monoexponential association ($^3H(t) = {}^3H_{max} * e^{-k*t}$) adapted to the time course of radioactivity released after the initial washout of the ^{3}H-BK labelling dose (120 s after termination of the infusion of labelled kinins). The total amount of radioactivity released during this period or retained in the myocardium at the end of perfusion was regarded as myocardial uptake that is given in relation to the total ^{3}H-activity for each experiment. All quantitative data are given as mean ± s.e.mean of the indicated number of independent experiments. The influence of kininase inhibition on the correlation between magnitude and duration of vasodilation was tested by comparing the linear regression of both parameters using analysis of covariance. Further comparisons were analysed using Student's *t*-test or analysis of variance with Newman-Keuls *post-hoc* test, as appropriate. Differences were considered to be statistically significant at an error level of $P < 0.05$.

Results

After the initial recovery of isolated perfused hearts, their basal perfusion pressures stabilized at an average level of 104 ± 4 mmHg. Consecutive 1 min applications of BK induced dose-dependent vasodilation ($EC_{50} = 5.1 \pm 0.8$ nM, $n = 12$) leading to acute reductions in perfusion pressures of up to 53 ± 6 mmHg. As reported earlier (Dendorfer *et al*., 2000), the inhibition of ACE and APP by ramiprilat and mercaptoethanol acutely decreased perfusion pressure and prevented the restoration of baseline pressure after high BK doses, so that the maximum change in perfusion pressure was diminished to 32 ± 3 mmHg. The maximum vasodilation during BK application was not influenced by kininase inhibition (62 ± 3 mmHg vs 55 ± 4 mmHg minimum perfusion pressure, $P > 0.05$). The half-maximally effective dose of BK was lowered by factor 14 under this condition ($EC_{50} = 0.36 \pm 0.11$ nM, $n = 9$). After termination of BK infusion, the re-adaptation of perfusion pressure could be described using monoexponential kinetics whereby the half-lives correlated well with the extent of vasodilation provoked at different doses (Figure 1). This correlation was significantly displaced to a longer persistence of vasodilation after equieffective BK doses when kinin degradation was inhibited (Figure 1). The maximum half-life of vasodilation under control conditions (112 ± 20 s, $n = 11$) was increased to 398 ± 130 s ($n = 9$) when ramiprilat and mercaptoethanol were applied ($P < 0.05$). The statistically significant difference in the correlations of vasodilation and its persistence was still apparent when the reductions in perfusion pressure had been normalized in order to compensate for the reduction in maximum efficacy during kininase inhibition.

In a second experiment, we asked whether the increased persistence of vasodilation after equieffective BK doses could be attributed to a prolonged receptor occupation or to new receptor associations occurring despite the termination of BK infusion. To answer this, two sequential stimulations (30 nM BK, 1 min) of isolated perfused hearts were performed in the presence of kininase inhibitors, whereby HOE 140 (100 nM) was infused immediately after termination of the second BK application to block unoccupied B_2-receptors. The efficacy of both stimulations was identical (60 ± 7 and 59 ± 7 mmHg pressure reduction) and the long duration of vasodilation during kininase inhibition observed for sequentially increasing doses in the first experiment was also confirmed for single applications (half-life 150 ± 21 s, Figure 2). However, the prolonged action of BK during kininase inhibition was prevented when BK's actions were terminated by B_2-blockade (half-life 74 ± 13 s, $P < 0.05$, $n = 5$, Figure 2). This action of the B_2-antagonist suggested that the prolongation of BK

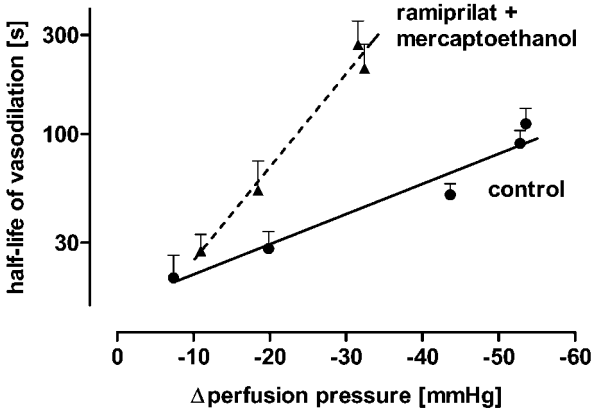

Figure 1 Influence of kininase inhibition on the persistance of BK-induced vasodilation. Various doses of BK were sequentially administered to isolated hearts for 1 min periods under control conditions and after inhibition of ACE and aminopeptidase P by ramiprilat (250 nM) and mercaptoethanol (1 mM). The acute vascular response is given as the drop in perfusion pressure during BK infusion. The persistence of vasodilation after termination of BK application is reflected by the half-life of perfusion pressure restoration. The correlation of both parameters is depicted for BK concentrations of 1 – 100 nM under control conditions, and for 0.1 – 3 nM during kininase inhibition. The slopes of linear regression and the maximum half-lives under both conditions differed significantly ($P < 0.05$, $n = 9 – 12$).

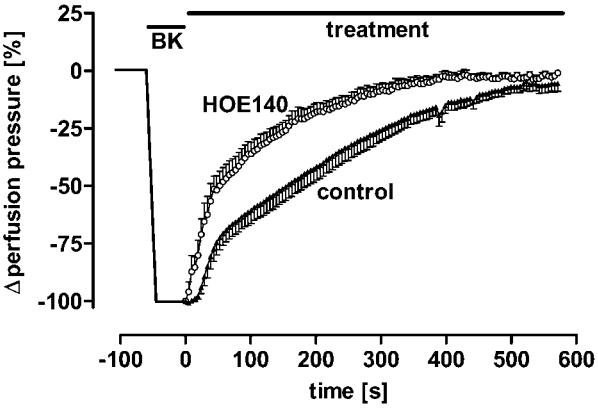

Figure 2 Persistence of BK-induced vasodilation is reduced by B_2-receptor blockade during kininase inhibition. Vasodilation was induced with two 1 min applications of 30 nM BK, the second of which was followed by immediate infusion of the B_2-receptor antagonist HOE 140 (100 nM). The half-life of pressure restoration during kininase inhibition (150 ± 21 s) was reduced by the subsequent application of HOE 140 (74 ± 13 s) ($P < 0.05$, $n = 5$). This duration was equivalent to the persistence of BK effects in the absence of kininase inhibitors (half-life 90 ± 14 s, $n = 12$, data not depicted).

effects by kininase inhibitors does not involve an alteration of receptor binding, but that it is based rather on new and therefore at least in part 'repeated' associations of BK with B_2-receptors even after termination of BK application.

The prolongation of BK's actions due to repeated receptor associations indicated a possible retainment of BK in the myocardium. This was studied in isolated perfused rat hearts labelled for 1 min with tritiated BK or a tritiated B_2-antagonist (^{3}H-NPC 17731) by determining the elution kinetics of radioactivity over the following 15 min. A comparison of the release of ^{3}H-activity in the absence and presence of kininase inhibitors is depicted in Figure 3. Consistent with a prolonged action of BK during application of ramiprilat and mercaptoethanol, ^{3}H-BK was also more effectively retained in the myocardium under these conditions. The total radioactivity that was retained for longer than 120 s (i.e. that was released after that time or present in the myocardium at the end of perfusion) amounted to $1.7 \pm 0.24\%$ of the applied dose under control conditions. This myocardial uptake was increased to $7.7 \pm 2.6\%$ during treatment with kininase inhibitors ($P < 0.05$, $n = 5$, Figure 3). The enhanced efficacy of kinin uptake during kininase inhibition was abolished when B_2-receptors were blocked by HOE 140 (100 nM) throughout the labelling period (uptake $2.0 \pm 0.34\%$ of the applied dose, $P < 0.05$ vs ^{3}H-BK during kininase inhibition, $n = 5$), or when ^{3}H-BK had been substituted by the B_2-antagonist ^{3}H-NPC 17731 ($3.4 \pm 0.34\%$ of the applied dose, $P < 0.05$ vs ^{3}H-BK during kininase inhibition, $n = 5$). The half-lives of ^{3}H-release under all conditions (range 70 – 141 s) were similar in duration as the functional persistence of vasodilation, but showed no significant differences among the treatment groups.

Because of the vasodilatory efficacy of incorporated BK, the compartment responsible for kinin uptake must be endowed with B_2-receptors and must be associated with endothelial cells. With the aim of localizing this myocardial compartment we immuno-labelled B_2-receptors on ultrathin sections and checked the cellular distribution of B_2-receptors by transmission electron microscopy (Figure 4). Weak staining was generally observed at cardiomyocyte membranes and at the free basal and apical surfaces of endothelial cells. The highest density of immuno-labelling was apparent in the distinct regions where clusters of membrane-attached and cytosolic vesicles filled the whole depth of the endothelial cells. In these areas, gold label was present predominantly at the apical membrane and at the endothelial vesicles. The uniform size distribution of these vesicles suggests their identification as endothelial caveolae.

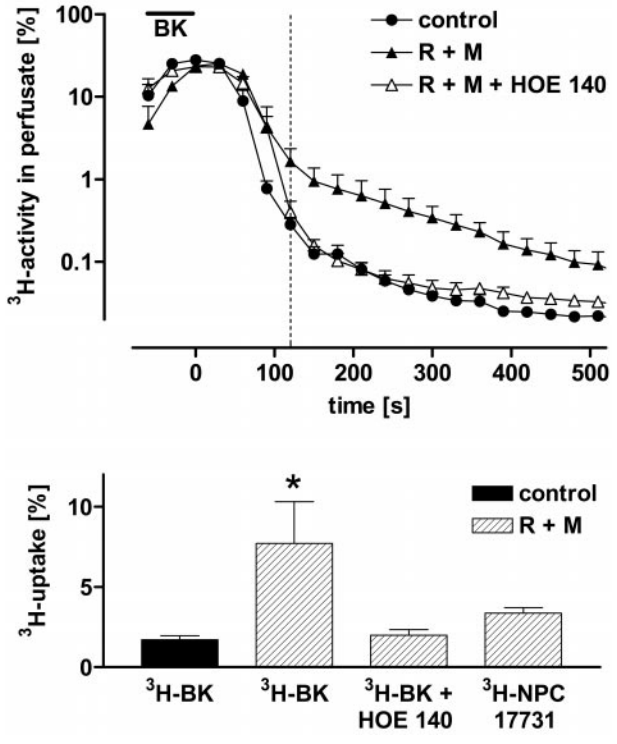

Figure 3 Myocardial uptake of ^{3}H-kinins. Perfused hearts were labelled for 1 min periods with 2 pmol of either ^{3}H-BK or the B_2-receptor antagonist ^{3}H-NPC 17731. The release of ^{3}H-activity into the perfusate was followed in 30 s intervals (upper graph). The total radioactivity released from the heart 120 s after termination of ^{3}H-kinin infusion (dotted line) or contained in the myocardium at the end of perfusion, was considered as myocardial uptake. Inhibition of kinin degradation with ramiprilat and mercaptoethanol (R + M) considerably increased ^{3}H-BK uptake, an effect that was prevented by concomitant treatment with HOE 140 (lower graph). The B_2-antagonist ^{3}H-NPC 17731 was not retained by the heart to an extent comparable to ^{3}H-BK. This indicates the involvement of B_2-receptor stimulation in the enhancement of kinin uptake during kininase inhibition. (*$P < 0.05$ vs all other groups, $n = 5$).

Discussion

This study demonstrated in the rat heart that the decay of BK-induced vasodilation after termination of BK application is delayed when higher doses of BK are used for stimulation.

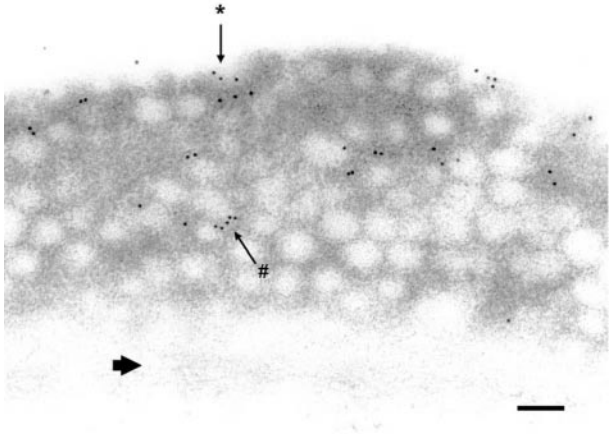

Figure 4 Localization of B_2-receptors in endothelial membrane vesicles, as demonstrated by post-embedding immunoelectron microscopy. B_2-receptors were labelled with specific polyclonal antibodies that were visualized by a secondary antibody conjugated to 6 nm gold particles. The micrograph shows a cross-section of a capillary endothelial cell attached to the basal membrane (arrow). The highest densities of B_2-receptors were found in distinct regions of endothelial cells where abundant membrane vesicles were present. B_2-receptors were identified in invaginated pits of the luminal cell membrane (*) as well as in cytoplasmic vesicles (#). The requirement of mild fixation conditions did not permit the identification of these vesicles, however their size and distribution were consistent with those of caveolae (bar represents 100 nm).

This phenomenon is not explained by first order receptor kinetics, but it may indicate a local storage of the peptide. This hypothesis is supported by the finding that inhibition of BK degradation *via* ACE and APP does not just increase the potency of BK, but that it also prolongs the persistence of vasodilation in an independent manner. Consequently, kinin degradation appears not only to limit the availability of BK at endothelial B_2-receptors, but also to contribute significantly to the inactivation of locally trapped peptide.

This theory is strengthened in two ways by the present study. Firstly, it could be demonstrated that B_2-receptors become newly activated, and consequently associate with BK, even when the application of BK had been terminated. Such an interpretation presumes that HOE 140 attains inhibitory concentrations in the B_2-receptor compartment more rapidly than BK would normally be eliminated. This kinetic is produced in the present study by applying HOE 140 at molar excess compared to BK. Since the B_2-receptor affinity of HOE 140 is in the order of 5 nM (Hock *et al*., 1991), the presence of only 5% of the applied concentration at the B_2-receptor sites should exert inhibitory activity. When the mean rate of BK efflux (half-life 100 s) is also applied to the invasion of HOE 140 into the B_2-receptor compartment, this inhibitory concentration should be established within about 7 s ($-\ln 0.95/\ln 2^* 100$ s). As such, the access of HOE 140 to B_2-receptors should be sufficiently rapid to explain the observed termination of BK activity in terms of an acute inhibition of new receptor associations.

A second indication of the proposed B_2-receptor compartment came from the confirmation of uptake and retention of BK in the myocardium using tritiated kinin tracers. The presence of BK in this compartment during kininase inhibition (half-life 93 s) corresponded well with the persistence of BK actions (150 s). The amount of retained BK was substantially increased by kininase inhibitors, an effect which was not accompanied by a substantial alteration in the kinetics of ^{3}H-release from the myocardium. As such, kininase inhibition increases efficacy and rate of BK uptake during the labelling period rather than prolonging the storage of the tracer. The suppression of BK uptake by HOE 140 and the significantly lower uptake activity towards the B_2-antagonist NPC 17731 indicates the essential role of B_2-receptor stimulation for myocardial BK uptake. The slightly higher uptake of NPC 17731 in comparison to BK ($P>0.05$) may be explained by its partial agonistic activity (Fathy *et al*., 1999) that appears to foster the spontaneous internalization of this substance after having associated with B_2-receptors (Houle *et al*., 2000). It should also be noted that the protracted release of radioactivity from the heart can be expected to predominantly consist of labelled BK degradation products. Bradykinin retained in the myocardium for as short as 30 s had previously been found to be degraded by more than 92% (Dendorfer *et al*., 1997). Since kinin levels in the perfusate will not reflect the condition in which BK is taken up and reaches the B_2 receptors, analysis of kinin levels in the perfusate of the present experiments was not further pursued.

The significance of kinin breakdown for BK uptake and for endothelial vasodilation indicates that the compartment of BK uptake colocalizes both, kininases and B_2-receptors, and that it must be in contact with the vascular endothelium. This study provides morphological evidence that such a compartment may be constituted by endothelial membrane vesicles. A more detailed identification of these vesicles was not attempted. However, various properties of caveolae would indicate their suitability to establish the described functional compartment. Caveolae are omega-shaped membrane invaginations which can detach from the plasma membrane to form discrete vesicles within the cell cytoplasm. Caveolae have been implicated in a variety of signal transduction processes including transport and regulation of receptors, and in the local association of receptors with hormones and secondary messenger components (reviewed by Gumbleton *et al*., 2000). In line with the interpretation of the present study, the presence of ACE and of B_2-receptors has been demonstrated in endothelial caveolae by morphological and biochemical means (Benzing *et al*., 1999; de Weerd & Leeb-Lundberg, 1997; Haasemann *et al*., 1998; Reiner *et al*., 2001; Ryan *et al*., 1975).

Caveolae may also be involved in the receptor-mediated uptake of kinins observed in our study since their role in the agonist-induced sequestration and internalization of B_2-receptors is well documented (Benzing *et al*., 1999; de Weerd & Leeb-Lundberg, 1997; Haasemann *et al*., 1998). However, this phenomenon has been linked to the desensitization of B_2-receptors and is not believed to be capable of delivering receptors and ligand back to the cell surface within a few minutes (Munoz & Leeb-Lundberg, 1992). On the other hand, caveolae may also form rapidly exchanging distribution compartments. Caveolae are able to cycle dynamically between an accessible invaginated and a closed vesicular state thereby providing a receptor-mediated temporal and spatial control of ligand binding to intracaveolar receptors (Anderson *et al*., 1992; Smart *et al*., 1995). In addition, caveolar vesicles in endothelial cells are arranged in fused clusters which communicate with the cell surface and with each other so that they have been implicated in the transcellular transport of solute molecules (Noguchi *et al*., 1987; Predescu *et al*., 1997; Schnitzer *et al*., 1994). As such, a kinin-induced increase in endothelial transcellular permeability (Schaeffer *et al*., 1993) may be based on the fusion and transport of caveolae which conjointly increase the local distribution volume for BK and thus promote its retention.

Further support for the formation of a functional B_2-receptor compartment by caveolae can be derived from their significance in B_2-receptor signal transduction. Endothelial NO synthase is inhibited by its association with either caveolin-1 or B_2-receptors (Ju *et al*., 1998). Stimulation of B_2-receptors induces their dimerization with STAT3 to a complex that is translocated out of caveolae as an initial step of signal transduction (Ju *et al*., 2000). This distribution is paralleled by that of NO synthase which also abandons caveolae in a kinin-dependent manner (Reiner *et al*., 2001). The basal residence of responsive B_2-receptors in caveolae has been unambiguously shown in isolated endothelial caveolae which preserve the functional coupling of B_2-receptors and NO synthase, both of which can be activated by BK *in vitro* (Chambliss *et al*., 2000).

Although caveolae appear to be a prominent candidate for the formation of a B_2-receptor compartment, their identity is still ambiguous. In the rat heart, APP is equivalent to ACE with respect to the degradation and to the functional potentiation of kinins (Dendorfer *et al*., 2000). However, APP is anchored by a glycosylphosphatidylinositol (GPI) moiety, which favours a distribution to cholesterol-rich lipid rafts rather than to caveolae. Furthermore, potentiation of

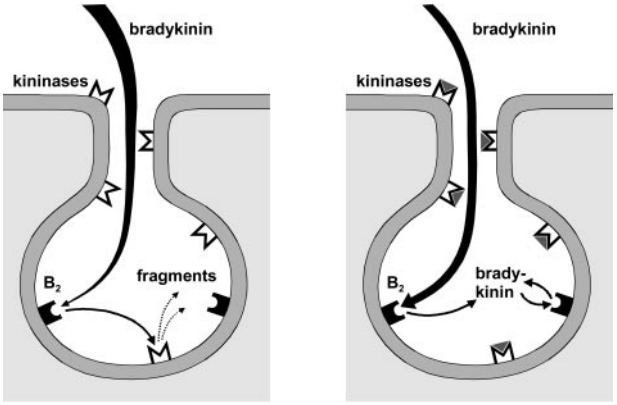

Figure 5 Schematic representation of the proposed interactions between the local distribution, degradation, and activity of BK. The extensive potentiation and prolongation of BK effects by kininase inhibitors can be explained by a colocalization of B_2-receptors and kininases in a distinct distribution compartment. The diffusion and metabolic barriers of this compartment limit the availability of BK at the B_2-receptor sites. Under basal conditions, the uptake of BK does not prolong its activity because of rapid local metabolism of free BK (left figure). In contrast, the activity of retained BK is preserved after inhibition of kinin breakdown, thus enabling repeated associations of BK with B_2-receptors and a prolongation of its effects (right figure).

BK actions in porcine coronary arteries by quinaprilat has been found to be insensitive to the caveolae-disrupting agents filipin and cyclodextrine (Tom *et al*., 2002). As such, caveolae are only one possibility for the formation of an endothelial compartment containing B_2-receptors, and further membrane structures (e.g. lipid rafts, coated pits, or the recently proposed vesiculo-vacuolar organelle (Dvorak & Feng, 2001)) may have similar properties. It may even be conceived that the observed uptake of BK corresponds to an extravasation into the interstitial space which may be enhanced in an agonist-dependent manner by an increase in endothelial permeability. This possibility seemed unlikely, since a very high exchange rate was determined for the interstitial compartment (half-life 12 s (Dendorfer *et al*., 2000)) which does not correspond to the kinetics of retention and action of BK demonstrated in the present study.

A distinction between ACE and APP was not attempted in the present study, since it was aimed at the functional evaluation of kinin breakdown in general. A similar significance of each enzyme may be presumed since in the rat heart, ACE and APP are equivalent with regard to their quantitative contribution to kinin degradation, to their intravascular (e.g. endothelial) localization, and to their functional efficacy in the attenuation of BK-induced vasodilation (Dendorfer *et al*., 1997; 2000). The occurrence of the kinin fragments [1-7]-, [1-5]- and [2-9]-BK in the cardiac perfusate under comparable conditions is in accordance with the crucial role of ACE and APP in myocardial kinin degradation (Dendorfer *et al*., 1997). As an additional kinin degradation pathway, the activity of neutral endopeptidase (E.C. 24.11) has been identified in rat myocardium. Under our experimental conditions, this enzyme accounts for about 3% of total kinin degradation and is virtually absent at the endothelium (Dendorfer *et al*., 1997). A similar situation appears to exist in porcine coronary arteries, where inhibition of neutral endopeptidase was shown to be ineffective in potentiating the vasodilatory response to BK (Tom *et al*., 2002). Since vasodilation, and most likely kinin uptake as well, reflect kinin actions at the endothelium exclusively, a special consideration of neutral endopeptidase in our study appeared to be dispensable. In addition, the use of a third enzyme inhibitor would have increased the risk of causing non-specific side effects.

With regard to the selectivity and efficacy of the kininase inhibitors used, mercaptoethanol may appear as a rather nonselective substance. However, at the concentration used, it is specific for APP, it does not influence cardiac performance or perfusion, it potentiates BK actions in a manner correlating to the inhibition of degradation, and most importantly, it exerts no degradation-independent influence on the B_2-mediated vasodilation provoked by stable kinin analogues (Dendorfer *et al*., 1997; 2000; Orawski *et al*., 1989). Since comparable data were not available for the alternative APP inhibitor apstatin, and in order to keep consistent with the conditions of previous studies, mercaptoethanol was the preferred choice as an inhibitor of APP.

The findings of the present study permit the formulation of a new hypothesis to explain the highly effective potentiation and independent prolongation of kinin effects produced by kininase inhibitors solely on the basis of a local kinin accumulation (illustrated in Figure 5). Due to a colocalization of B_2-receptors and kininases in an endothelial membrane compartment, BK comes into close contact with local kininases during diffusion to the receptor site, while its availability and potency is greatly reduced by degradation. After dissociation of BK from B_2-receptors, its actions are terminated due to the rapid breakdown by closely associated kininases. Inhibition of kinin degradation preserves retained BK in its active state thereby enabling its repeated associations with B_2-receptors. Inhibition of BK breakdown enhances BK actions not only by increasing its local availability but also by promoting the uptake of BK into the B_2-receptor compartment.

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References

ANDERSON, R.G., KAMEN, B.A., ROTHBERG, K.G. & LACEY, S.W. (1992). Potocytosis: sequestration and transport of small molecules by caveolae. *Science,* **255,** 410–411.

AUCH-SCHWELK, W., BOSSALLER, C., CLAUS, M., GRAF, K., GRÄFE, M. & FLECK, E. (1993). ACE inhibitors are endothelium dependent vasodilators of coronary arteries during submaximal stimulation with bradykinin. *Cardiovasc. Res.,* **27,** 312–317.

BENZING, T., FLEMING, I., BLAUKAT, A., MÜLLER-ESTERL, W. & BUSSE, R. (1999). Angiotensin-converting enzyme inhibitor ramiprilat interferes with the sequestration of the B_2 kinin receptor within the plasma membrane of native endothelial cells. *Circulation,* **99,** 2034–2040.

CHAMBLISS, K.L., YUHANNA, I.S., MINEO, C., LIU, P., GERMAN, Z., SHERMAN, T.S., MENDELSOHN, M.E., ANDERSON, R.G. & SHAUL, P.W. (2000). Estrogen receptor alpha and endothelial nitric oxide synthase are organized into a functional signaling module in caveolae. *Circ. Res.,* **87,** E44 – E52.

DE WEERD, W.F. & LEEB-LUNDBERG, L.M. (1997). Bradykinin sequesters B_2 bradykinin receptors and the receptor-coupled Galpha subunits Galphaq and Galphai in caveolae in DDT1 MF-2 smooth muscle cells. *J. Biol. Chem.,* **272,** 17858 – 17866.

DENDORFER, A., REISSMANN, S., WOLFRUM, S., RAASCH, W. & DOMINIAK, P. (2001). Potentiation of kinin analogues by ramiprilat is exclusively related to their degradation. *Hypertension,* **38,** 142 – 146.

DENDORFER, A., WOLFRUM, S., SCHÄFER, U., STEWART, J.M., INAMURA, N. & DOMINIAK, P. (2000). Potentiation of the vascular response to kinins by inhibition of myocardial kininases. *Hypertension,* **35,** 32 – 37.

DENDORFER, A., WOLFRUM, S., WELLHÖNER, P., KORSMAN, K. & DOMINIAK, P. (1997). Intravascular and interstitial degradation of bradykinin in isolated perfused rat heart. *Br. J. Pharmacol.,* **122,** 1179 – 1187.

DVORAK, A.M. & FENG, D. (2001). The vesiculo-vacuolar organelle (VVO). A new endothelial cell permeability organelle. *J. Histochem. Cytochem.,* **49,** 419 – 432.

FATHY, D.B., LEEB, T., MATHIS, S.A. & LEEB-LUNDBERG, L.M. (1999). Spontaneous human B_2 bradykinin receptor activity determines the action of partial agonists as agonists or inverse agonists. Effect of basal desensitization. *J. Biol. Chem.,* **274,** 29603 – 29606.

FIGUEROA, C.D., GONZALEZ, C.B., GRIGORIEV, S., ABD ALLA, S.A., HAASEMANN, M., JARNAGIN, K. & MÜLLER-ESTERL, W. (1995). Probing for the bradykinin B_2 receptor in rat kidney by anti-peptide and anti-ligand antibodies. *J. Histochem. Cytochem.,* **43,** 137 – 148.

GOBEIL JR. F., HALLE, S., BLAIS, P.A. & REGOLI, D. (2002). Studies on the angiotensin-converting enzyme and the kinin B_2 receptor in the rabbit jugular vein: modulation of contractile response to bradykinin. *Can. J. Physiol. Pharmacol.,* **80,** 153 – 163.

GUMBLETON, M., ABULROB, A.G. & CAMPBELL, L. (2000). Caveolae: an alternative membrane transport compartment. *Pharm. Res.,* **17,** 1035 – 1048.

HAASEMANN, M., CARTAUD, J., MÜLLER-ESTERL, W. & DUNIA, I. (1998). Agonist induced redistribution of bradykinin B_2 receptor in caveolae. *J. Cell Sci.,* **111,** 917 – 928.

HECKER, M., BLAUKAT, A., BARA, A.T., MÜLLER-ESTERL, W. & BUSSE, R. (1997). ACE inhibitor potentiation of bradykinin-induced venoconstriction. *Br. J. Pharmacol.,* **121,** 1475 – 1481.

HERZIG, M.C. & LEEB-LUNDBERG, L.M. (1995). The agonist binding site on the bovine bradykinin B_2 receptor is adjacent to a sulfhydryl and is differentiated from the antagonist binding site by chemical cross-linking. *J. Biol. Chem.,* **270,** 20591 – 20598.

HOCK, F.J., WIRTH, K., ALBUS, U., LINZ, W., GERHARDS, H.J., WIEMER, G., HENKE, S., BREIPOHL, G., KONIG, W. & KNOLLE, J. (1991). Hoe 140 a new potent and long acting bradykinin-antagonist: in vitro studies. *Br. J. Pharmacol.,* **102,** 769 – 773.

HOULE, S., LARRIVÉE, J.F, BACHVAROVA, M., BOUTHILLIER, J., BACHVAROV, D.R. & MARCEAU, F. (2000). Antagonist-induced intracellular sequestration of rabbit bradykinin B_2 receptor. *Hypertension,* **35,** 1319 – 1325.

JU, H., VENEMA, V.J., LIANG, H., HARRIS, M.B., ZOU, R. & VENEMA, R.C. (2000). Bradykinin activates the Janus-activated kinase/signal transducers and activators of transcription (JAK/STAT) pathway in vascular endothelial cells: localization of JAK/STAT signalling proteins in plasmalemmal caveolae. *Biochem. J.,* **351,** 257 – 264.

JU, H., VENEMA, V.J., MARRERO, M.B. & VENEMA, R.C. (1998). Inhibitory interactions of the bradykinin B_2 receptor with endothelial nitric-oxide synthase. *J. Biol. Chem.,* **273,** 24025 – 24029.

LINZ, W., WIEMER, G., GOHLKE, P., UNGER, T. & SCHÖLKENS, B.A. (1995). Contribution of kinins to the cardiovascular actions of angiotensin-converting enzyme inhibitors. *Pharmacol. Rev.,* **47,** 25 – 49.

MARCIC, B., DEDDISH, P.A., SKIDGEL, R.A., ERDÖS, E.G., MINSHALL, R.D. & TAN, F. (2000). Replacement of the transmembrane anchor in angiotensin I-converting enzyme (ACE) with a glycosylphosphatidylinositol tail affects activation of the B_2 bradykinin receptor by ACE inhibitors. *J. Biol. Chem.,* **275,** 16110 – 16118.

MINSHALL, R.D., TAN, F., NAKAMURA, F., RABITO, S.F., BECKER, R.P., MARCIC, B. & ERDÖS, E.G. (1997). Potentiation of the actions of bradykinin by angiotensin I-converting enzyme inhibitors. The role of expressed human bradykinin B_2 receptors and angiotensin I-converting enzyme in CHO cells. *Circ. Res.,* **81,** 848 – 856.

MUNOZ, C.M. & LEEB-LUNDBERG, L.M. (1992). Receptor-mediated internalization of bradykinin. DDT1 MF-2 smooth muscle cells process internalized bradykinin via multiple degradative pathways. *J. Biol. Chem.,* **267,** 303 – 309.

NARDONE, J. & HOGAN, P.G. (1994). Delineation of a region in the B_2 bradykinin receptor that is essential for high-affinity agonist binding. *Proc. Natl. Acad. Sci. U.S.A,* **91,** 4417 – 4421.

NOGUCHI, Y., SHIBATA, Y. & YAMAMOTO, T. (1987). Endothelial vesicular system in rapid-frozen muscle capillaries revealed by serial sectioning and deep etching. *Anat. Rec.,* **217,** 355 – 360.

ORAWSKI, A.T., SUSZ, J.P. & SIMMONS, W.H. (1989). Metabolism of bradykinin by multiple coexisting membrane-bound peptidases in lung: techniques for investigating the role of each peptidase using specific inhibitors. *Adv. Exp. Med. Biol.,* **247B,** 355 – 364.

PREDESCU, S.A., PREDESCU, D.N. & PALADE, G.E. (1997). Plasmalemmal vesicles function as transcytotic carriers for small proteins in the continuous endothelium. *Am. J. Physiol,* **272,** H937 – H949.

REINER, M., BLOCH, W. & ADDICKS, K. (2001). Functional interaction of caveolin-1 and eNOS in myocardial capillary endothelium revealed by immunoelectron microscopy. *J. Histochem. Cytochem.,* **49,** 1605 – 1610.

RYAN, J.W., RYAN, U.S., SCHULTZ, D.R., WHITAKER, C. & CHUNG, A. (1975). Subcellular localization of pulmonary antiotensin-converting enzyme (kininase II). *Biochem. J.,* **146,** 497 – 499.

SCHAEFFER JR. R.C., GONG, F., BITRICK JR. M.S. & SMITH, T.L. (1993). Thrombin and bradykinin initiate discrete endothelial solute permeability mechanisms. *Am. J. Physiol.,* **264,** H1798 – H1809.

SCHNITZER, J.E., OH, P., PINNEY, E. & ALLARD, J. (1994). Filipin-sensitive caveolae-mediated transport in endothelium: reduced transcytosis, scavenger endocytosis, and capillary permeability of select macromolecules. *J. Cell Biol.,* **127,** 1217 – 1232.

SMART, E.J., YING, Y.S. & ANDERSON, R.G. (1995). Hormonal regulation of caveolae internalization. *J. Cell Biol.,* **131,** 929 – 938.

TOM, B., DENDORFER, A., DE VRIES, R., SAXENA, P.R. & DANSER, A.H. (2002). Bradykinin potentiation by ACE inhibitors: A matter of metabolism. *Br. J. Pharmacol.,* **137,** 276 – 284.

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