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Novel Mode of Action of Angiotensin I Converting Enzyme Inhibitors

DIRECT ACTIVATION OF BRADYKININ B_1 RECEPTOR*

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Angiotensin I converting enzyme (kininase II; ACE) inhibitors are important therapeutic agents widely used for treatment in cardiovascular and renal diseases. They inhibit angiotensin II release and bradykinin inactivation; these actions do not explain completely the clinical benefits. We found that enalaprilat and other ACE inhibitors in nanomolar concentrations activate human bradykinin B_1 receptors directly in the absence of ACE and the B_1 agonist des-Arg^{10}-Lys^1-bradykinin. These inhibitors activate at the Zn^{2+}-binding consensus sequence HE*XX*H (195–199) in B_1, which is present also in ACE but not in the B_2 receptor. Activation elevates $[Ca^{2+}]_i$ and releases NO from endothelial or transfected cells expressing the B_1 receptor but is blocked by Ca-EDTA, a B_1 receptor antagonist, the synthetic undecapeptide sequence (192–202) of B_1, and the mutagenesis of His^{195} to Ala^{195}. Except for the B_1 antagonist, these agents and manipulations did not block activation by a peptide ligand. Thus, Zn^{2+} is essential for B_1 receptor activation by ACE inhibitors at the zinc-binding consensus sequence. Ischemia or cytokines induce abundant B_1 receptor expression. B_1 receptor activation by ACE inhibitors, a novel mode of action reported here first, can contribute to their therapeutic effects by releasing NO in the heart and to some side effects.

Angiotensin I converting enzyme inhibitors (ACEIs)[1] are used for treatment in conditions such as hypertension, congestive heart failure, diabetic nephropathy, and others (1–8). For instance, the administration of an ACEI after a myocardial infarction and in the absence of any thrombolytic therapy reduced the incidence of death or the development of severe congestive heart failure (3, 4). ACEIs were also reported to inhibit neointima formation after endothelial injury (7). Despite the beneficial effects of ACEI therapy proven in many millions of patients world-wide, the modes of action of ACEIs have not been fully characterized (5). The inhibition of ACE or kininase II blocks angiotensin II release or bradykinin (BK) inactivation (9–11), but these actions alone do not fully explain their effectiveness (5). ACEIs also potentiate the effects of BK and its ACE-resistant analogs on their B_2 receptor by inducing an enzyme/receptor protein-protein interaction (12–14), a heterodimer formation (14).

Of the two BK receptors B_1 and B_2, B_2 is widely expressed and primarily mediates the actions of kinins under physiological conditions (11). Normally, few cell types express the B_1 receptor, but various pathologic conditions such as ischemia, atheromatous disease, or exposure to inflammatory cytokines rapidly induce expression (15, 16). The elimination of the B_2 receptor gene in knockout mice also up-regulated the B_1 receptor (17).

The ligands of the two receptors differ, as plasma carboxypeptidase N or tissue carboxypeptidase M cleave the C-terminal Arg of the B_2 receptor agonists kallidin (Lys-BK) and BK to generate B_1 agonists des-Arg-kinins (18). Of the products, des-Arg^{10}-kallidin (des-Arg^{10}-Lys^1-bradykinin) is about three orders of magnitude more potent than des-Arg^9-BK on the B_1 receptor (15).

The contributions of the kinin B_2 receptor to the effects of ACEIs have been established (10, 13), and a possible but not previously explored role for the B_1 receptor could be deduced from the fact that many patients treated with ACEIs suffer from conditions that lead to B_1-receptor induction (15).

Although ACEIs do not directly affect the BK B_2 receptor, they augment its function when ACE is also expressed on the cell surface (12–14). Here we report that ACEIs in nanomolar concentrations directly activate the BK B_1 receptor in cells without an intermediate peptide ligand and in the absence of ACE. We also characterized the site and the mode of action of ACEIs on the human B_1 receptor, resulting in the generation of nitric oxide (NO).

MATERIALS AND METHODS

Cell Culture and Transfection—Chinese hamster ovary (CHO) cells (ATCC, Manassas, VA) were grown as described (14). Human embryonic kidney (HEK 293) and COS-7 cells (ATCC) were cultured using Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and an antibiotic solution diluted 1:100. Bovine pulmonary arterial endothelial (BPAE) cells were cultured following manufacturer's instructions (BioWhittaker, Walkersville, MD). The medium of human fetal lung fibroblasts (IMR-90) (19) (ATCC) was supplemented with 15% fetal bovine serum.

CHO cells were stably transfected with cDNA of the human B_1 receptor inserted into pcDNA3 (donated by Dr. F. Leeb-Lundberg of the University of Texas, San Antonio) using SuperFect essentially as described for the human B_2 receptor (14). HEK 293 or COS-7 cells were transiently transfected with the wild type or H195A mutant human B_1 receptor using SuperFect or LipofectAMINE 2000 as described by the manufacturer (Qiagen, Valencia, CA and Invitrogen). Experiments were done 24 h after transfection.

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[1] The abbreviations used are: ACEI, angiotensin I converting enzyme inhibitor; ACE, angiotensin I converting enzyme; BK, bradykinin; kallidin, Lys-Bk; CHO, Chinese hamster ovary; HEK 293, human embryonic kidney; BPAE, bovine pulmonary arterial endothelial; WT, wild type.

Membrane Preparation—HEK 293 cells were transiently transfected with the wild type cDNA of the human B_1 receptor, as above, and the plasma membrane fraction was obtained with a slight modification of the technique in Ref. 20.

Radioligand Binding—Binding assays were performed at room temperature with [^{3}H]des-Arg^{10}-kallidin in the presence and absence of enalaprilat concentrations ranging from 0.1 nM to 10 μM (modified from Ref. 20).

Use of Inhibitors—(Ethylenedinitrilo)tetraacetic acid dicalcium salt (Ca-EDTA) was added to the cells for 30 min at 1 mM concentration to bind Zn^{2+}, then cells were washed with zinc-free medium. The undecapeptide (LLPHEAWHFAR) was synthesized by the Protein Sciences Facility (University of Illinois, Champaign) and tested as an inhibitor (10 or 100 μM) after 20 min of preequilibration.

Measurement of Changes in Intracellular Free Ca^{2+} ($[Ca^{2+}]_i$)—$[Ca^{2+}]_i$ was measured using the Ca^{2+}-sensitive fluorescent probe fura-2 AM in a PTI Deltascan (Princeton, NJ) or Attofluor RatioVision (Islandia, NY) instrument (14). Fura-2 fluorescence was detected at 510 nm following excitation at 340 and 380 nm, and the ratio of intensities at 340 and 380 nm were recorded in 10–100 cells simultaneously (12–14).

Site-directed Mutagenesis—The H195A mutation of the human B_1 receptor was done by the PCR method using a QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA). The human wild type B_1 pcDNA3 was used as the template with the two mutagenic primers shown in the following sequences: B1HA$_1$, 5′-CTGCTCCTCCCCGCTGAGGCCTGGCACTTT and B1HA$_2$, 5′-GTGCCAGGCCTCAGCGGGGAGGAGCAGGAT. The sequence of the construct was confirmed by automatic sequencing at the DNA core facility of the University of Illinois, Chicago.

Detection of Nitric Oxide—NO was measured using either a porphyrinic microsensor (21, 22) or with a fluorescence assay (23) in BPAE cells. The microsensor consists of carbon fibers which are electroplated with a highly conductive polymeric porphyrin to facilitate the electron transfer on or from NO to the sensor. Cells were preincubated for a few minutes at 37 °C until a stable baseline was established. Ligands were added, and the responses (current *versus* time) were recorded continuously. Current generated on porphyrinic electrode was proportional to the NO released and was quantitated with a known standard NO solution.

For the fluorescence assay we used diamino fluorescein diacetate. Cellular fluorescence was measured at 515 nm following excitation at 490 nm (23).

Statistics—Statistical analysis was performed using one-way analysis of variance test.

RESULTS

Effect of Enalaprilat on IMR-90 Human Fetal Lung Fibroblasts—To investigate whether ACEIs can activate the BK B_1 receptor, we tested them on IMR-90 fibroblasts that constitutively express both the B_1 and B_2 receptors. As a prototype of an active ACEI we used enalaprilat and measured the increase in $[Ca^{2+}]_i$ from cells (Fig. 1). The receptors were activated with either BK for the B_2 receptor or des-Arg^{10}-kallidin as a ligand of the B_1 receptor. BK (10 nM) and des-Arg^{10}-kallidin (10 nM) raised $[Ca^{2+}]_i$ in distinctly different patterns (Fig. 1, *A* and *B*). B_1 receptor activation led to a very prolonged, sustained increase in $[Ca^{2+}]_i$, whereas BK stimulated a more transient response. Enalaprilat (1 nM) in the absence of a peptide agonist significantly enhanced the $[Ca^{2+}]_i$ level (Fig. 1*C*). Enalaprilat mediated a response which clearly differed temporally from the one caused by BK, but was very similar to that produced by des-Arg^{10}-kallidin. These experiments indicated that enalaprilat in nanomolar concentrations directly activated the B_1 receptor within seconds in the absence of added kinins. Using B_1 and B_2 receptor antagonists, des-Arg^{10}-Leu^9-kallidin for B_1 and HOE 140 for B_2 (Fig. 1, *D* and *E*), we confirmed this conclusion because the response to enalaprilat was completely inhibited by the B_1 antagonist, whereas HOE 140 had no effect.

Effect of Enalaprilat on the Transfected Human B_1 Receptor in CHO Cells—The data with IMR-90 cells strongly suggested that the B_1 receptor was mediating the direct effect of enalaprilat. However, because IMR-90 cells have low but detectable

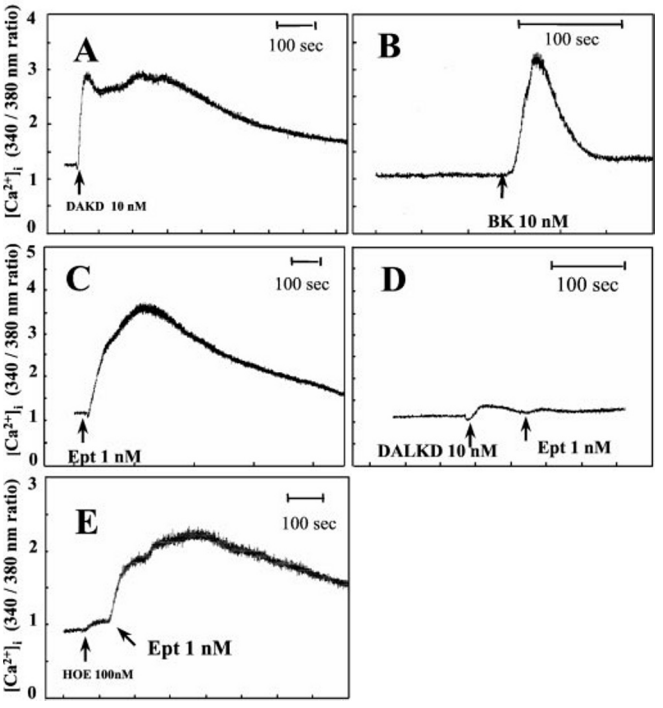

FIG. 1. **Stimulation of $[Ca^{2+}]_i$ transients in IMR-90 fibroblasts.** IMR-90 cells were stimulated with des-Arg^{10}-kallidin (*DAKD*) (*A*), BK (*B*), or enalaprilat (*Ept*) (*C*). Notice that the effect of enalaprilat was blocked by des-Arg^{10}-Leu^9-kallidin (*DALKD*), the B_1 receptor antagonist (*D*), but not by HOE 140, the B_2 receptor antagonist (*E*). *Arrows* denote the time of addition of agonists or antagonists. Representative experiments were repeated two to four times with 10–100 cells in each assay.

ACE activity, we had to exclude the possibility that ACE is required for the activation of the B_1 receptor, similar to the B_2 receptor (12). Therefore, CHO cells, having no ACE (12), were transfected to express human B_1 receptors (CHO/B_1). Adding enalaprilat (10 nM) caused immediately, within seconds, a typical B_1 receptor agonist response as indicated by the prolonged shape of the elevated $[Ca^{2+}]_i$ curve (Fig. 2, *A* and *B*). As control, we also tested transfected CHO cells expressing ACE only (CHO/ACE) or the B_2 receptor (CHO/B_2) with negative results; thus, enalaprilat, in the absence of the B_1 receptor, was inactive in these cells (Fig. 2, *C–E*).

Enalaprilat Stimulates Nitric Oxide Release in Endothelial Cells—Enalaprilat also elevated $[Ca^{2+}]_i$ in BPAE cells, which constitutively express both the B_1 and the B_2 receptor (24) (not shown). We determined whether the enalaprilat-induced elevation of $[Ca^{2+}]_i$ levels would stimulate NO production. We monitored the release of NO from cultured BPAE cells using either a fluorescence assay or an electrochemical method with a porphyrinic microsensor (see "Materials and Methods"). Enalaprilat (10 nM) did indeed stimulate NO release, and this response was more prolonged than the transient one that followed B_2 receptor activation (Fig. 3*A*). Its pattern resembled the prolonged elevation of $[Ca^{2+}]_i$ (Fig. 1*C*) and mimicked the response of the cells to the B_1 agonist des-Arg^{10}-kallidin (Fig. 3*A*). The dose-response curve showed that enalaprilat in log M concentrations linearly enhanced the release of NO from 10 nM to 10 μM (Fig. 3*B*). We repeated these experiments with another technique to measure NO release based on fluorescence detection and obtained similar results (not shown).

Enalaprilat stimulated NO production by BPAE cells via the B_1 receptor. This conclusion is based on findings that enalaprilat and des-Arg^{10}-kallidin released NO similarly (242 nM ± 5 S.E. per well and 288 ± 7 nM NO), and the B_1 receptor antagonist des-Arg^{10}-Leu^9-kallidin (100 nM) reduced both effects

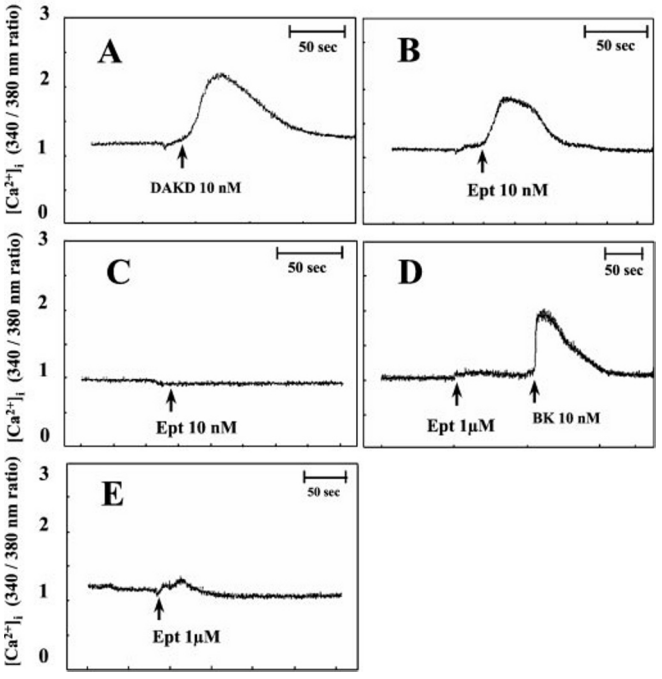

FIG. 2. **Enalaprilat activates the human wild type B_1 receptor transfected into CHO cells.** The increase in $[Ca^{2+}]_i$ levels over time in CHO/B_1 cells stimulated with des-Arg^{10}-kallidin (*DAKD*) (*A*) or enalaprilat (*Ept*) (*B*), which was added at the time indicated by the *arrows*, is shown. Results are representative of six independent experiments. Enalaprilat was inactive in native CHO cells (*C*), CHO/B_2 cells, which responded to BK (*D*), and in CHO/ACE cells (*E*). Results are representative of four or more independent experiments.

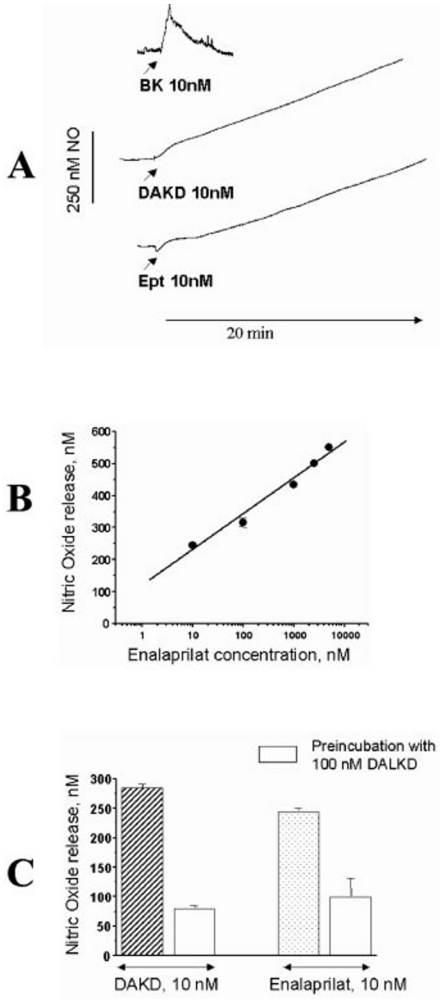

FIG. 3. **Stimulation of NO generation.** NO production was measured in BPAE cells using a porphyrinic microsensor in real time. *A*, the addition of enalaprilat (*Ept*) or des-Arg^{10}-kallidin (*DAKD*) (denoted by the *arrows*) caused an immediate generation of NO, which continued to increase over 20 min. In contrast, BK stimulated a transient increase in NO, which returned to base line by about 5 min. *B*, the dose-response curve for enalaprilat is shown. BPAE cells were stimulated with increasing concentrations of enalaprilat and the NO concentration generated at 20 min taken as a measure of the response. Results represent the mean values of five (10 and 100 nM) or two (μM concentration points) independent experiments. *C*, the B_1 receptor blocker inhibits enalaprilat stimulation of NO production. Des-Arg^{10}-kallidin and enalaprilat were added to cells pretreated for 2 min with or without the B_1 receptor antagonist (*DALKD*) as indicated, and the NO concentration generated at 20 min was taken as a measure of the response. Shown are the mean values from four or more independent experiments.

(101 ± 26 and 81 ± 4 nM) for enalaprilat and des-Arg^{10}-kallidin (Fig. 3*C*; $p < 0.01$, $n = 4$–5).

Search for the Site of Activation—To establish where ACEIs activate the B_1 receptor, we compared the amino acid sequence of ACE with the BK B_1 and B_2 receptors (15, 25–27). Although there is little overall homology, the human B_1 receptor contains in its second extracellular loop (residues 195–199) an HEAWH sequence (Fig. 4*A*), and this is similar to the one in the active centers of the two domains of ACE (HEMGH) (26, 28) and matches the HE*XX*H zinc-binding consensus sequence (29) in other members of the zinc metalloproteases. This motif is absent from the B_2 receptor. The ACEIs combine with the active centers of ACE via the Zn^{2+} cofactor with their SH or COO^- group, and mutation of the HE*XX*H motif eliminates inhibitor binding (30); thus, this region in B_1 receptor was very likely the site for activation by inhibitors.

[H195A]B_1 Receptor Mutant—To investigate the importance of that lead, we constructed a point mutation (H195A) at the putative Zn-binding site (HEAWHA→AEAWH). The [H195A] B_1 receptor mutant was transiently expressed in HEK 293 cells. In HEK/[H195A]B_1 cells, des-Arg^{10}-kallidin activated the receptor, whereas enalaprilat was inactive (Fig. 4*B*). In HEK 293 control cells transfected to express the wild type (WT) B_1 receptor, 10 nM des-Arg^{10}-kallidin or enalaprilat elevated the $[Ca^{2+}]_i$ level in a typical B_1 receptor pattern. Transfecting COS-7 cells with [WT]B_1 or [H195A]B_1 gave similar results (not shown; $n = 3$). Mock transfected or untransfected cells (both HEK 293 and COS-7) in the absence of the B_1 receptor did not respond to either des-Arg^{10}-kallidin or enalaprilat (not shown). Consequently, the HEAWH sequence in the second extracellular loop is essential for the direct activation of the B_1 receptor by enalaprilat but not for des-Arg^{10}-kallidin, which acts on other epitopes in the third extracellular loop (20, 31).

To explore the role of Zn^{2+} on receptor response, we preincubated BPAE cells for 30 min with 1 mM Ca-EDTA. This heavy metal sequestering agent blocked the effect of 10 nM enalaprilat completely ($n = 4$), whereas 10 nM des-Arg^{10}-kallidin ($n = 5$) raised the level of $[Ca^{2+}]_i$ uninhibitedly. Enalapril (the inactive prodrug of enalaprilat), which has an esterified carboxyl group that does not bind Zn^{2+}, did not activate the B_1 receptor in IMR-90 cells at 1 μM, ($n = 3$) or 10 nM concentration. We concluded that for activation by a peptide ligand zinc is not needed, but it is essential for activation by enalaprilat.

Blockade by B_1-(192–202) Undecapeptide—To further confirm the importance of our finding obtained with the [H195A]B_1 mutant, we used the synthetic undecapeptide (LL-PHEAWHFAR) corresponding to residues 192–202 of the B_1 receptor, the putative site of activation by ACEIs. The presence of 10 or 100 μM of this peptide blocked the effect of the ACEI completely, whereas des-Arg^{10}-kallidin was not affected (Fig. 5; $n = 3$). As control, the same concentration of another peptide

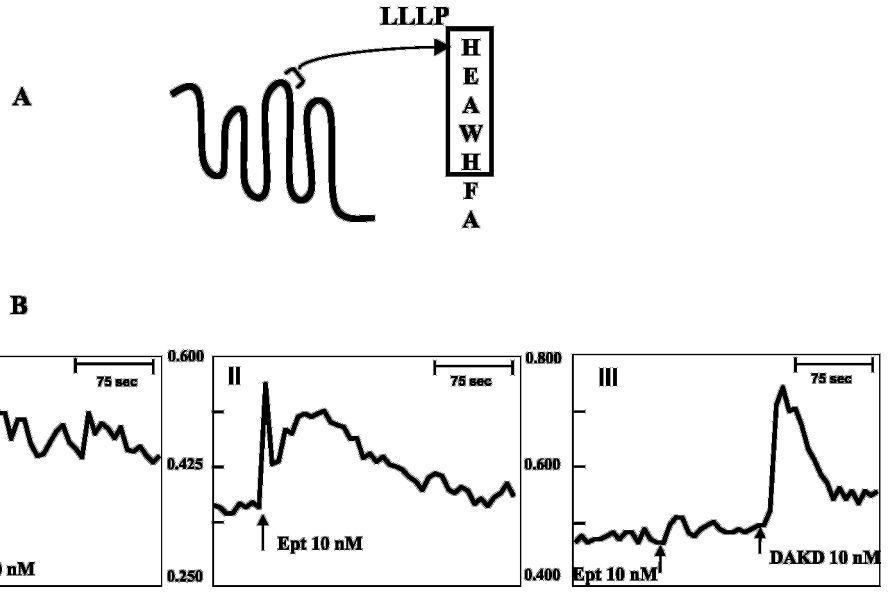

FIG. 4. **Site of the activation of B_1 by enalaprilat.** *A*, a *schematic* of the structure of the human B_1 receptor, redrawn from Ref. 15, is shown. The *HEAWH* motif in the second extracellular loop (residues 195–199), the proposed binding site for enalaprilat, was enlarged. This putative zinc-binding sequence (HEAWH) is well conserved in B_1 receptors across species. *B*, tracings from single HEK cells expressing [WT]B_1 (*panels I* and *II*) or the [H195A]B_1 mutant (*panel III*) are shown. The cells were stimulated with des-Arg^{10}-kallidin (*DAKD*) and enalaprilat (*Ept*). Notice that the H195A mutation of the B_1 receptor in the Zn-binding region abolished only the effect of enalaprilat, whereas des-Arg^{10}-kallidin remained active. Results are representative of five independent experiments.

(AIKLGTGRRFTTC) of similar size but unrelated sequence affected neither enalaprilat nor des-Arg^{10}-kallidin responses in the BPAE cells ($n = 3$, not shown).

Competition Binding—Our experiments strongly suggested that ACEIs and des-Arg^{10}-kallidin bind at different sites of the B_1 receptor. We investigated this further in competition binding assays. Membrane preparations, obtained from homogenized HEK 293 cells and transiently transfected with the wild type B_1 receptor, were exposed to 1 nM [^{3}H]-des-Arg^{10}-kallidin in the absence or presence of increasing concentrations of enalaprilat. Enalaprilat competed with des-Arg^{10}-kallidin binding and replaced the labeled ligand at the B_1 receptor but only at a relatively high (>1 μM) concentration (Fig. 6). This is in contrast with the ability of nanomolar enalaprilat to release NO or raise $[Ca^{2+}]_i$ but is in agreement with the conclusion that des-Arg^{10}-kallidin and enalaprilat activate the B_1 receptor at different sites.

Other ACEIs—We also tested other ACEIs to determine whether the direct B_1 receptor activation is specific to enalaprilat or is rather a group-related effect. Like enalaprilat, captopril (10 nM, $n = 4$ and 1 μM, $n = 2$) and ramiprilat (1 nM, 10 nM and 1 μM, $n = 3$) elevated $[Ca^{2+}]_i$ levels by activating the B_1 receptor in BPAE cells. On the other hand, another active but structurally different ACEI, lisinopril (10 nM, $n = 5$; 1 μM, $n = 3$), did not activate the B_1 receptor in these cells (data not shown).

We also tested D-penicillamine (α-amino-β-methyl-β-mercaptobutyric acid), which is structurally related to captopril. D-penicillamine was inactive on the B_1 receptor in BPAE cells (10 nM, $n = 3$; 1 μM, $n = 3$; 100 μM, $n = 2$). Also, bradykinin-potentiating pentapeptide (BPP5a), an ACEI (32, 33) and a slowly cleaved substrate (9), was inactive in 100 nM and 10 μM concentrations ($n = 2, 5$; not shown).

DISCUSSION

Tens of millions of patients worldwide are treated with ACEIs (1–7). Because ACE has a dual action, it activates angiotensin I and inactivates BK by cleaving C-terminal dipeptides (9, 18, 28), and its inhibitors block the release of the potent vasoconstrictor and mitogen angiotensin II and augment activation of the B_2 receptor by BK. Besides preventing kinin inactivation by ACE (10), these inhibitors indirectly potentiate B_2 because they induce an ACE/B_2 receptor cross-talk (12–14). Des-Arg^{10}-kallidin is the endogenous ligand that potently activates the second kinin receptor, B_1, at a low concentration (15). The role of the B_1 receptor in contributing to ACEI therapy has not been explored systematically. We showed above that ACEIs in nanomolar concentrations directly activate the human or bovine B_1 receptor in cultured cells in the absence of ACE or peptide ligands at a different extracellular domain than the peptide. ACEIs were inactive on cells that did not express the BK B_1 receptor.

ACEIs release NO (34, 35), which contributes to their beneficial therapeutic effects. The mechanism of NO release by ACEIs is usually attributed to blocking BK inactivation and thereby potentiating the effect of BK on B_2 receptors, which stimulate endothelial NO synthesis (10). Activation of the B_1 receptors by peptide ligand also releases NO (16). Enalaprilat in nanomolar concentrations did indeed stimulate NO production from pulmonary arterial endothelial cells, and the prolonged release pattern was similar to that caused by des-Arg^{10}-kallidin but not by BK (Fig. 3). The effect of enalaprilat was suppressed by des-Arg^{10}-Leu^9-kallidin, a B_1 receptor antagonist, and NO production increased linearly with the log M concentration of the inhibitor.

We found the sequence in the B_1 receptor where enalaprilat activated directly after comparing the amino acid sequences of human ACE and BK B_2 and B_1 receptors. The second extracellular loop of the human B_1 receptor contains the HEAWH (195–199) sequence (15, 25). This HE*XX*H motif represents a Zn^{2+}-binding sequence of the two active centers in the N- and C-domains of ACE and in other members of the zincin family of zinc-metalloenzymes (29), but it is absent in the B_2 receptor (27).

The mutation of this Zn^{2+}-binding domain to remove an essential His residue abolished the effect of enalaprilat but not that of des-Arg^{10}-kallidin. In agreement with this finding, enalapril, the inactive prodrug with an esterified carboxyl group that does not bind zinc, did not activate the B_1 receptor. This

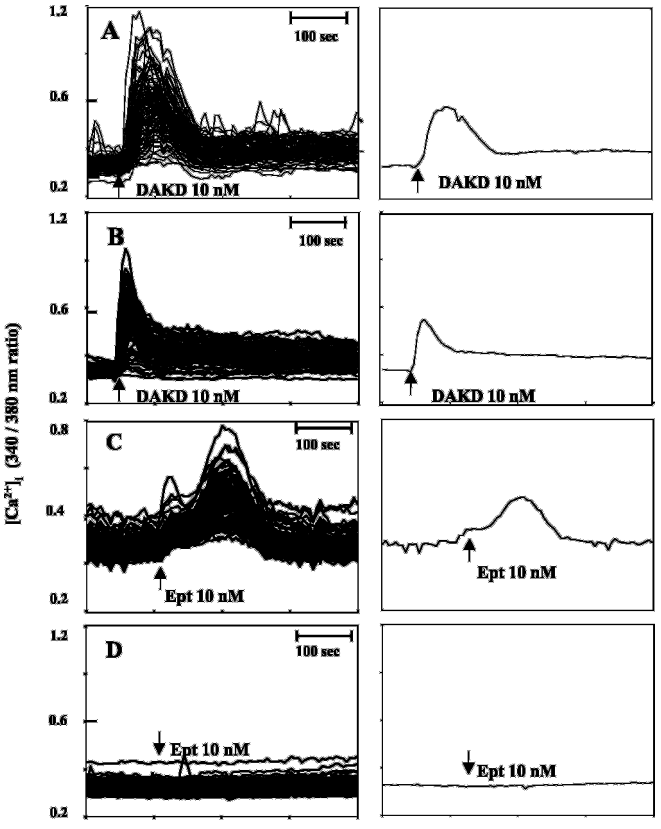

FIG. 5. **Effect of the synthetic undecapeptide LLPHEAWHFAR from the B_1 receptor Zn-binding sequence.** BPAE cells were stimulated with des-Arg^{10}-kallidin (*DAKD*) (*panels A* and *B*) or enalaprilat (*Ept*) (*panels C* and *D*). The *left side panels* represent individual tracings obtained from simultaneous measurement of up to 100 cells; on the *right side* the calculated mean values are shown. The cells in *panels B* and *D* were preincubated with the undecapeptide (10 μM) for 20 min prior to addition of des-Arg^{10}-kallidin or enalaprilat. Note that the added peptide blocked only the effect of enalaprilat but not that of des-Arg^{10}-kallidin (n = 3).

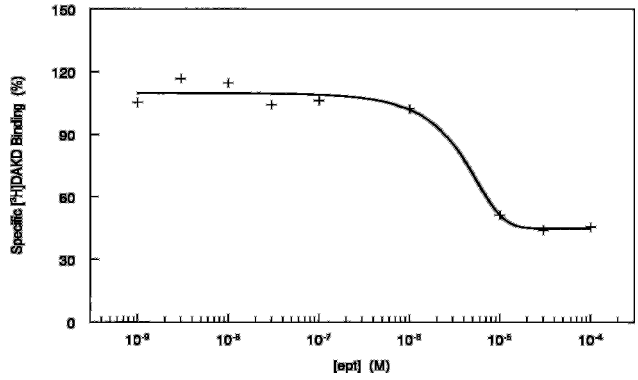

FIG. 6. **[^{3}H]des-Arg^{10}-kallidin competition binding with enalaprilat.** Membrane preparations from HEK/B_1 cells were incubated with [^{3}H]des-Arg^{10}-kallidin (*[^{3}H]DAKD*) in the presence of increasing concentrations of enalaprilat, and [^{3}H]des-Arg^{10}-kallidin binding was measured relative to that of membranes not treated with enalaprilat (taken as 100%). Results are representative of three independent experiments; all the points were assayed in triplicate.

was further supported by experiments where Ca-EDTA or a synthetic undecapeptide (LLPHEAWHFAR) that corresponds to sequence 192–202 of B_1 and incorporates the suggested site of activation blocked the effect of enalaprilat. Therefore, the HEAWH sequence in the second extracellular loop of the B_1 receptor is essential for the direct effect of enalaprilat on the B_1 receptor. The absence of a similar sequence in the B_2 receptor explains the lack of direct action of ACEIs on it (11–13). The B_1 receptor may belong to a group of receptors that is modulated by zinc ions (36).

The peptide inhibitor of ACE, BPP5a, is essentially a slow substrate (9) and did not activate the B_1 receptor. This is not surprising, as it is not expected of the B_1 receptor to hydrolyze ACE substrates. Of the ACEIs tested for a direct effect on the B_1 receptor, only lisinopril did not activate it. This consequently shows that ACE inhibition alone is not a sufficient attribute for a molecule to bind to the B_1 receptor. However, for this class of clinically used ACEIs, the ability to inhibit ACE seems to be necessary for B_1 receptor binding. The inactive prodrug enalapril, which only differs in structure from enalaprilat by an esterified carboxyl group, is inactive on the B_1 receptor. ACEIs are highly active (nanomolar concentration) agonists of B_1 receptors, also indicating that many of the features necessary to inhibit ACE potently are required of agonists that bind at the HEAWH receptor site. It is unlikely that a common structural element, unrelated to ACE inhibition, would link ACEIs to B_1 receptors. This conclusion is also supported by the fact that lisinopril was inactive on the B_1 receptor. The structure of lisinopril is identical to that of enalaprilat except for the presence of a lysine side chain in the P1′ position; enalaprilat and most of the other ACEIs have only a methyl group at this position in contrast to the $-(CH_2)_4$-NH_2 of lisinopril. Thus, the presence of this large charged substituent is likely to be the reason why lisinopril does not activate the B_1 receptor; it could prevent access to a recessed zinc-binding site, or the positively charged side chain could be repelled by a positive charge on the receptor (37).

ACEIs are used extensively with few side effects (1–8). The B_1 receptor can be rapidly induced under various pathological conditions (for example, by cytokines, ischemia and atherosclerosis) (15, 16) undoubtedly encountered by many subjects treated with ACEIs. A recent report (38), published after submission of this manuscript, showed that ACEIs themselves can induce expression of the B_1 receptor in rodent kidney, heart, and vasculature and that B_1 receptor stimulation plays a role in the hypotensive effect of ACEIs. Furthermore, the up-regulation is dependent on stimulation of the B_1 receptor itself because B_1 receptor antagonists blocked it (38). The mode of activation of the receptor at the cellular or molecular level was not investigated, but our results indicate it could be due to direct stimulation of the B_1 receptor by ACEIs. Activation of B_1 receptor by ACEIs could mediate some of the beneficial effects of these drugs by stimulating NO production. For example, administration of an ACEI shortly after acute myocardial infarction reduced the incidence of death or development of severe congestive heart failure (3, 4). Acute myocardial infarction also induces B_1 receptor expression (39). The induction and activation of the B_1 receptor could prove advantageous under various stressful conditions, ranging from infection to cardiovascular disorders (15). The B_1 receptor may protect the heart in ischemic preconditioning (40). Activation of B_1 receptors in an ischemic heart inhibited norepinephrine outflow and prevented potentially lethal ventricular fibrillation (41). Neointima formation after balloon angioplasty was suppressed by the B_1 receptor (42) and also by ACEIs after endothelial injury (7). The novel mode of action of ACEIs via B_1 receptor, described above, may add to the therapeutic effects of ACEIs (43) in other pathological conditions (44), and furthermore it may contribute to some side effects.

In conclusion, using cultured cells which express B_1 receptor either constitutively or after transfection we demonstrated a direct activation of the BK B_1 receptor by ACEIs and identified the site of action. To our knowledge, this is the first report showing a direct effect of ACEIs, apart from their effects on

ACE, that relates to the therapeutic efficacy of this important class of drugs.

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