0026-895X/04/6605-1310–1316$20.00
MOLECULAR PHARMACOLOGY
Vol. 66, No. 5
1990/1179094
Mol Pharmacol 66:1310–1316, 2004
Printed in U.S.A.

Kinin B_1 Receptors Stimulate Nitric Oxide Production in Endothelial Cells: Signaling Pathways Activated by Angiotensin I-Converting Enzyme Inhibitors and Peptide Ligands

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Received April 27, 2004; accepted August 3, 2004

ABSTRACT

We reported previously a novel mode of action of angiotensin I-converting enzyme (kininase II; ACE) inhibitors mediated through the direct activation of bradykinin B_1 receptor, independent of endogenous kinins or ACE (*J Biol Chem* **277:**16847–16852, 2002). We aimed to further clarify the mechanism of activation of B_1 receptor, which leads to prolonged nitric oxide (NO) release. The ACE inhibitor enalaprilat and the peptide ligand desArg^{10}-kallidin (in nanomolar concentrations) release NO by activating endothelial NO synthase (eNOS) in bovine and inducible NO synthase (iNOS) in stimulated human endothelial cells. The peptide and the ACE inhibitor ligands activate eNOS by facilitating different signaling pathways. DesArg^{10}-kallidin enhances inositol-phosphate generation and elevates $[Ca^{2+}]_i$ by first augmenting intracellular release and then the influx of extracellular Ca^{2+}. In contrast, enalaprilat stimulates only the influx of extracellular Ca^{2+} through rare earth-sensitive channels, and its effect is blocked by cholera toxin or protein kinase C inhibitors. In addition, unlike desArg^{10}-kallidin, enalaprilat can also release NO independent of Ca^{2+} in bovine endothelial cells. The inflammatory cytokines interleukin-1β and interferon-γ induce both B_1 receptor and iNOS in human endothelial cells. In contrast to eNOS, B_1 ligands activate iNOS similarly. Both desArg^{10}-kallidin and ACE inhibitors enhance arginine uptake and release NO independent of $[Ca^{2+}]_i$ elevation. This is the first report on the direct activation of B_1 receptor by ACE inhibitors in human endothelial cells. This interaction leads to prolonged NO release and possibly contributes to the documented benefits of the use of ACE inhibitors.

Bradykinin and related peptides (kinins) activate two types of G-protein coupled receptors: B_1 and B_2. B_2 is ubiquitous, and B_1 is generally expressed under pathological conditions, in which it may protect or be detrimental (McLean et al., 2000). B_2 receptors are activated by bradykinin and kallidin, whereas the B_1 receptor agonists are desArg^9-bradykinin and desArg^{10}-kallidin (McLean et al., 2000) generated by membrane or plasma carboxypeptidases, M or N, which cleave the C-terminal arginine of the peptides (Erdös and Skidgel, 1997).

In addition to these endogenous peptide ligands, inhibitors of angiotensin I-converting enzyme (kininase II; ACE) can also directly activate B_1 receptors in cultured cells (Ignjatovic et al., 2002). They activate through the HEAWH sequence in the second extracellular loop (residues 195–199 in human receptor). This sequence is conserved in B_1 receptors in several species, and it matches the consensus pentameric HEXXH sequence of the catalytic domains in ACE and other metalloenzymes required to couple the Zn^{2+} cofactor. Activation of B_1 receptors by ACE inhibitor leads to prolonged nitric oxide (NO) release from cultured endothelial cells (Ignjatovic et al., 2002), indicating that B_1 receptors contribute to the widely reported clinical beneficial effects of ACE inhibitors. ACE inhibitors are administered to tens of millions of patients worldwide to treat hypertension, myocardial infarction, heart failure, or diabetic nephropathy (Pfeffer, 2001).

Several mechanisms can dictate the mode of action of ACE inhibitors at the cellular and molecular level. By inhibiting ACE, ACE inhibitors not only suppress the conversion of angiotensin I to angiotensin II but also block the inactivation

These studies were supported by National Heart, Lung, and Blood Institute grants HL36473 and HL58118 and National Institute of Diabetes and Digestive and Kidney Diseases grant DK41431.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.104.001990.

ABBREVIATIONS: ACE, angiotensin I-converting enzyme; iNOS, inducible nitric-oxide synthase; eNOS, endothelial nitric-oxide synthase; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetrakis(acetoxymethyl ester); AM, acetoxymethyl ester; BPAE, bovine pulmonary artery endothelial; HLMVE, human lung microvascular endothelial; IP_3, inositol 1,4,5-trisphosphate; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; 1400W, *N*-(3(aminomethyl) benzyl) acetamidine dihydrochloride; HOE140, icatibant.

of bradykinin (Linz et al., 1995; Yang and Erdös, 1967; Yang et al., 1971) and other ACE substrates (Carretero and Rhaleb, 2001). In addition, ACE inhibitors also potentiate bradykinin effects (Minshall et al., 1997; Marcic et al., 2000) through an induced cross-talk between ACE and the bradykinin B_2 receptor (Erdös et al., 1999; Marcic et al., 2000), shown with cultured cells and bioassays.

As an important part of their effect, ACE inhibitors release NO (Zahler et al., 1999; Ignjatovic et al., 2002). They stimulate NO release either by direct activation of the B_1 receptor (Ignjatovic et al., 2002) or indirectly, through the potentiation of bradykinin effects on the B_2 receptor (Linz et al., 1995; Minshall et al., 1997; Marcic et al., 2000).

The aim of this study was to further clarify the consequences after ACE inhibitors directly activate the native B_1 receptors independent of endogenous kinins. We wished to explain how ACE inhibitors release NO via activation of the B_1 receptor. In addition, we also investigated whether the two B_1 receptor agonists, peptide desArg^{10}-kallidin and ACE inhibitor enalaprilat, which act through different domains on the receptor (Fathy et al., 2000; Ignjatovic et al., 2002), may stimulate different signaling pathways. We report that peptide ligand and ACE inhibitor stimulate NO release by different second-messenger pathways in bovine pulmonary artery endothelial (BPAE) cells. In contrast, in cytokine-treated human lung microvascular endothelial (HLMVE) cells, they stimulate NO production via inducible NO synthase (iNOS) apparently by the same signaling mechanisms.

Materials and Methods

Materials. DesArg^{10}-kallidin and desArg^{10}-Leu^9-kallidin were obtained from Sigma-Aldrich (St. Louis, MO) or Bachem Biosciences (King of Prussia, PA). Enalaprilat, the active form of the ACE inhibitor enalapril, was from Merck (Whitehouse Station, NJ) or Toronto Research Chemicals, Inc. (North York, ON, Canada). Lisinopril, ramiprilat (the active form of ramipril), and captopril were from Merck, Aventis (Strasbourg, France), and Bristol-Myers Squibb Co. (Princeton, NJ), respectively. Fura-2/AM was purchased from Molecular Probes (Eugene, OR). Fetal bovine serum was from Atlanta Biologicals (Norcross, GA). Interleukin-1β and interferon-γ were obtained from Calbiochem (San Diego, CA) and Invitrogen (Carlsbad, CA). L-[2,3,4,5-^{3}H]Arginine monohydrochloride and [*myo*-2-^{3}H]inositol were purchased from Amersham Biosciences Inc. (Piscataway, NJ). Cholera toxin was purchased from Calbiochem and Sigma-Aldrich. Phosphate-buffered saline was from Mediatech (Herndon, VA). NOS inhibitors 1400W and N^{ω}-nitro-L-arginine, protease inhibitor cocktail, BAPTA-AM, protein kinase C inhibitors, and other chemicals and reagents were from Sigma-Aldrich.

Cell Culture. BPAE and HLMVE cells were purchased from Cell Applications (San Diego, CA) and Cambrex Bio Science Baltimore, Inc. (Baltimore, MD). BPAE cells were cultured in DMEM containing 15% fetal bovine serum and were used in passages 3 to 8. HLMVE cells were cultured in endothelial growth medium-2 containing 10 ng/ml human epidermal growth factor, 5 ng/ml vascular endothelial growth factor, 2 ng/ml human fibroblast growth factor, 2 ng/ml insulin-like growth factor, 0.2 μg/ml ascorbic acid, 50 ng/ml gentamicin-amphotericin B, and 10% fetal bovine serum. HLMVE cells were used in passages 5 to 8, and they were routinely treated with 5 ng/ml interleukin-1β and 50 ng/ml interferon-γ for 16 to 18 h before the experiment to induce B_1 receptors.

Human embryonic lung fibroblasts (IMR-90) were purchased from American Type Culture Collection (Manassas, VA) and were grown in DMEM supplemented with 15% fetal bovine serum, essential amino acid solution (1:100), and glutamine (2 mM).

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured with the Ca^{2+}-sensitive fluorescent probe fura-2/AM as described previously (Ignjatovic et al., 2002). The cells were grown to confluence on glass coverslips and then incubated for 45 min at 37°C in phenol-red free DMEM/Ham's F-12 medium containing fura-2/AM (2.5 μM). After the loading, cells were washed and mounted at room temperature in a Sykes-Moore chamber (Bellco, Vineland, NJ) on an inverted microscope coupled to the microspectrofluorometer PTI Deltascan (Princeton, NJ) or Attofluor Ratiovision (Islandia, NY) (Ignjatovic et al., 2002). Fura-2 fluorescence was detected at 510 nm after excitation at 340 and 380 nm. Ionophore was routinely used as positive control. The tracings show mean values obtained with the simultaneous measurement of changes in the $[Ca^{2+}]_i$ level in 10 to 100 cells. The results are shown as the ratio of intensities at 340 and 380 nm, which reflects the amount of free calcium in the cytosol.

Phosphoinositide Hydrolysis. Cells were plated in 12-well dishes and labeled in medium containing 5% fetal bovine serum with [^{3}H]inositol (2 μCi/ml) for 16 to 24 h. The following day, they were washed and then incubated in assay medium containing 5 mM LiCl for 10 to 15 min. After removing the medium, fresh assay medium containing agonists was added. After 20 min, medium was aspirated, cells extracted with formic acid (20 mM), and inositol phosphates separated by anion exchange chromatography (Seuwen et al., 1990). Results are expressed as the ratio of inositol phosphates/inositol phosphates + inositol counts.

NO. NO was measured electrochemically with porphyrinic microsensor as described previously (Ignjatovic et al., 2002). Cells were plated in 12-well plates and used upon reaching confluence in 2 days. The microsensor was positioned close to the cell surface, ligands were added, and the responses (current versus time) were recorded continuously. The current generated was proportional to the NO released, and a computer-based Camry VP600 potentiostat was used to monitor NO concentration in real time. All experiments were done at 37°C, and the baseline values were subtracted from the results obtained after the activation of the B_1 receptors.

L-Arginine Transport. Confluent cells plated in 24-well dishes were washed and assayed using L-[2,3,4,5-^{3}H]arginine monohydrochloride (diluted to 1 μCi/ml in DMEM/F-12 medium). After 60-s stimulation with desArg^{10}-kallidin or enalaprilat, the uptake was terminated by aspiration of the medium and rapid washing with ice-cold phosphate-buffered saline. The cells were solubilized with ice-cold 0.2% sodium dodecyl sulfate, and aliquots were counted with a Beckman LS6500 scintillation counter (Beckman Coulter, Fullerton, CA).

Statistical Analysis. Data were analyzed by Student's t test. Values were considered significant if $p < 0.05$.

Results

To study the mechanisms and consequences of B_1 receptor activation, we used two types of endothelial cells: BPAE and HLMVE cells. The bovine cells express B_1 receptor constitutively (Ignjatovic et al., 2002), whereas the human cells express it mainly after stimulation with proinflammatory cytokines interleukin-1β and interferon-γ (see *Materials and Methods*). We used these systems to investigate whether the activation of the constitutively expressed B_1 receptors in BPAE cells and the induced B_1 receptors (HLMVE) would stimulate NO release differently and whether this would depend on the ligand used.

Activation of Constitutively Expressed B_1 Receptor in BPAE Cells. We reported that both the ACE inhibitor enalaprilat and the peptide ligand desArg^{10}-kallidin activate the B_1 receptor to release NO from cultured BPAE cells (Ignjatovic et al., 2002). To determine the contributions of different NOS isoforms to NO production we used inhibitors

relatively specific for endothelial NOS (eNOS; N^{ω}-nitro-L-arginine) or iNOS (1400W) as described previously (Alderton et al., 2001). Figure 1 summarizes the experiments in which we released NO by peptide ligand, desArg^{10}-kallidin, or by the ACE inhibitor enalaprilat in BPAE cells. As shown in Fig. 1, desArg^{10}-kallidin (100 nM) released 290 ± 15 nM NO at 20 min, which was inhibited only 7% by the iNOS inhibitor (2 μM 1400W) but 66% by the eNOS inhibitor (2 μM N^{ω}-nitro-L-arginine). Enalaprilat (100 nM) stimulated NO production similar to that of the peptide ligand, but this was inhibited 68% by N^{ω}-nitro-L-arginine and 40% by 1400W (Fig. 1). Thus, the mediator of the desArg^{10}-kallidin response is eNOS, whereas enalaprilat activates mainly eNOS but also iNOS to release NO.

The intracellular calcium chelator BAPTA suppressed NO release by 100 nM desArg^{10}-kallidin (75% inhibition) but had much less effect on the response to 100 nM enalaprilat (36%) (Fig. 2).

ACE Inhibitors Elevate $[Ca^{2+}]_i$ by Augmenting Ca^{2+} Entry. After desArg^{10}-kallidin or enalaprilat activate the constitutively expressed B_1 receptors in BPAE cells, the $[Ca^{2+}]_i$ level increases (Ignjatovic et al., 2002). To elucidate how B_1 receptor agonists elevate $[Ca^{2+}]_i$, we used rare earth ions (Gd^{3+}) or Ca^{2+}-free medium to block extracellular Ca^{2+} entry. Calcium-channel blocker Gd^{3+} (10 or 1 μM) inhibited only the effect of enalaprilat (10 nM) but not that of the peptide agonist desArg^{10}-kallidin (10 nM) (Fig. 3). In addition, enalaprilat was inactive in calcium-free medium, whereas desArg^{10}-kallidin still increased the $[Ca^{2+}]_i$ level although for a shorter time and without a sustained elevated phase that usually follows the initial peak (Fig. 3).

The differences in eliciting $[Ca^{2+}]_i$ elevation by the two B_1 receptor agonists were further demonstrated by the experiments shown in Fig. 4. Activation of the B_1 receptors by ACE inhibitor did not release inositol-phosphates from BPAE cells, but the peptide ligand desArg^{10}-kallidin (10 nM) did. Human embryonic lung fibroblasts IMR-90 cells, which also constitutively express the B_1 receptor, stimulated by ACE inhibitors (Ignjatovic et al., 2002), yielded similar results (data not shown; $n = 3$). In addition to enalaprilat, two other ACE inhibitors, ramiprilat (10 nM and 1 μM) and captopril (10 nM and 1 μM), which activate the B_1 receptor to increase $[Ca^{2+}]_i$ (Ignjatovic et al., 2002) also did not stimulate [^{3}H]inositol phosphate production in BPAE cells (data not shown). Therefore, the lack of [^{3}H]inositol phosphate release is a shared characteristic of ACE inhibitors stimulating the B_1 receptor.

Effect of Cholera Toxin. We used toxins to test whether the stimulation of B_1 receptor by one or the other agonist can facilitate the coupling of different G proteins. Pretreatment of BPAE cells with cholera toxin (1 μg/ml for 20 h) abolished the effect of enalaprilat but not that of desArg^{10}-kallidin (Fig. 5) ($n = 4$–7). On the other hand, protein kinase A inhibitor (20 μM, 15–25 min preincubation) did not block the enalaprilat response (data not shown). We verified the activity of protein kinase A inhibitor in separate control experiments (data not shown) in which it inhibited forskolin-stimulated NO release (Ferro et al., 1999). These results indicate that activation of B_1 receptors by enalaprilat initiates a $G_{\alpha s}$-dependent but protein kinase A-independent pathway.

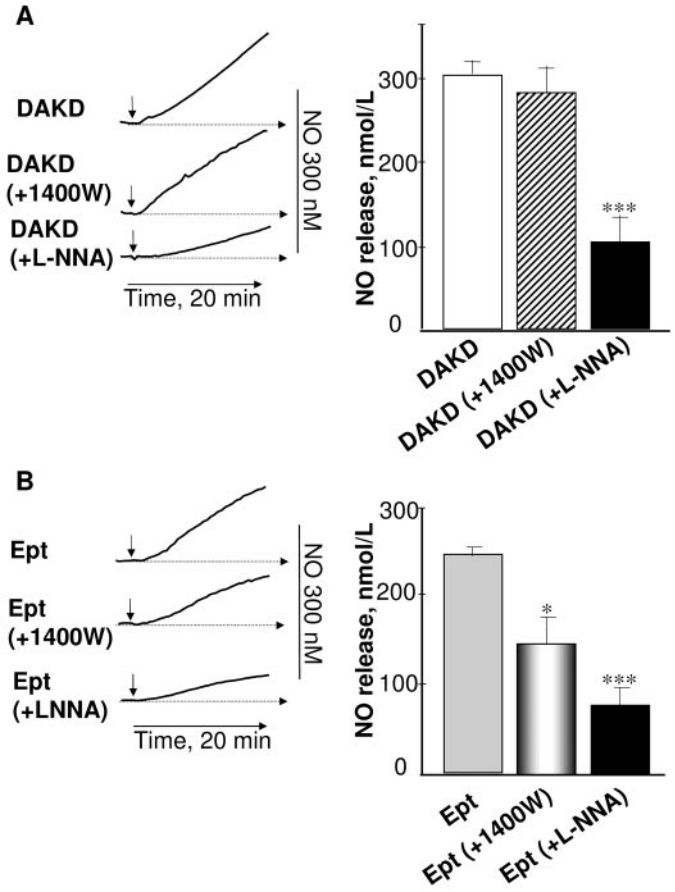

Fig. 1. NO release from BPAE cells. BPAE cells were preincubated (for 15 min at 37°C) with 2 μM N^{ω}-nitro-L-arginine (L-NNA) or 2 μM 1400W, and NO production in response to B_1 agonists was measured with a porphyrinic electrode. A, left, representative real-time tracings of NO production obtained with desArg^{10}-kallidin (DAKD; 100 nM) in the absence or presence of eNOS or iNOS inhibitors L-NNA or 1400W. Right, calculated mean values (± S.E.) from four to five experiments. Vertical bars show the response to standard 300 nM concentration of NO. B, representative tracings and mean values obtained with enalaprilat (Ept; 100 nM) in the absence and presence of eNOS and iNOS inhibitors ($n = 4$–5). ⋆, $p < 0.05$, significantly different from control; ⋆⋆⋆, $p < 0.001$, significantly different from control

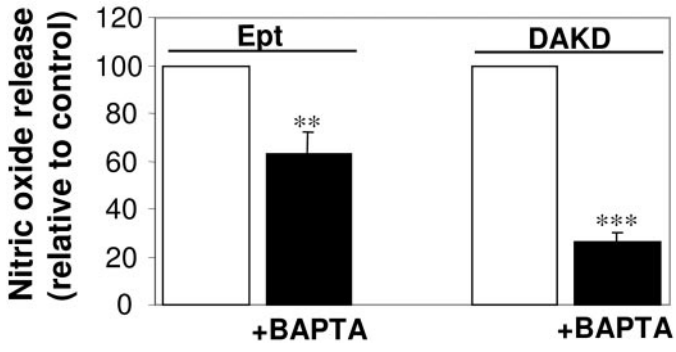

Fig. 2. The intracellular calcium chelator BAPTA inhibits NO release from BPAE cells. Cells were stimulated with enalaprilat (Ept; 100 nM) or desArg^{10}-kallidin (DAKD; 100 nM) in the absence or presence of BAPTA. ■, responses obtained in cells pretreated with BAPTA-AM (25 μM, 30 min). Abscissa shows the results relative to controls taken as 100%. Enalaprilat released 234 ± 11 or 147 ± 24 nM NO in control and BAPTA-pretreated cells, respectively. DesArg^{10}-kallidin released 270 ± 16 or 71 ± 11 nM NO in control and BAPTA-pretreated cells, respectively. Data shown are means ± S.E. from five experiments. ⋆⋆, $p < 0.01$, significantly different from control; ⋆⋆⋆, $p < 0.001$, significantly different from control.

Protein Kinase C and $[Ca^{2+}]_i$ Level. The protein kinase C inhibitor calphostin C (1 μM), after 15 min of preincubation, blocked the elevation of $[Ca^{2+}]_i$ by ACE inhibitors when tested in BPAE cells (Fig. 5C) but not that stimulated by desArg^{10}-kallidin (Fig. 5D) (n = 6–7). Another protein kinase C inhibitor, chelerythrine chloride (1 μM), after 30 min of preincubation yielded similar results (data not shown; n = 2).

Activation of Induced B_1 Receptor in HLMVE Cells. In addition to transfected and constitutively expressed B_1 receptors (Ignjatovic et al., 2002), we wanted to establish whether the ACE inhibitor enalaprilat activates induced B_1 receptor in stimulated HLMVE cells. In contrast to BPAE, the human endothelial cells tested had little or no constitutively expressed B_1 receptors. We stimulated them with interleukin-1β and interferon-γ for 16 to 18 h before the experiments to induce B_1 receptor expression (see *Materials and Methods*). Interleukin-1β stimulates the expression of both B_1 receptor (Schanstra et al., 1998; McLean et al., 2000; Sangsree et al., 2003) and the iNOS isoform (Asano et al., 1994; Kanno et al., 1994; Kolyada and Madias, 2001), whose activity is not Ca^{2+}-regulated.

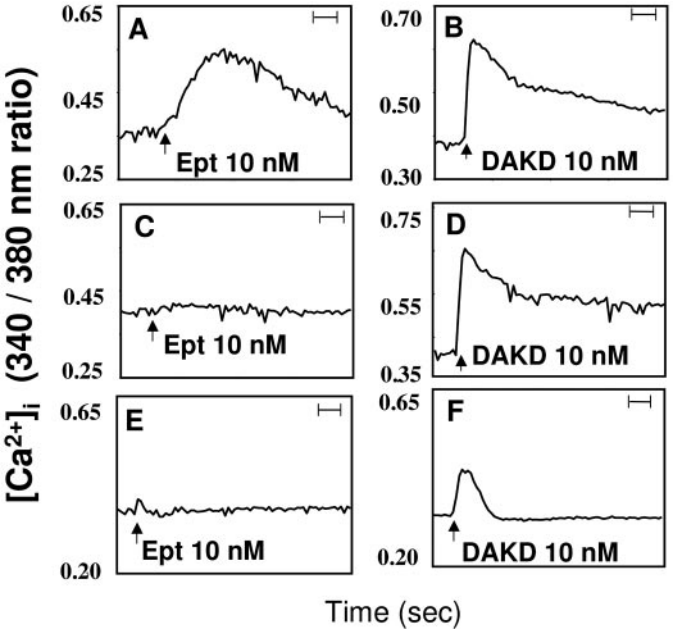

Fig. 3. ACE inhibitors increase intracellular calcium via calcium entry. BPAE cells were stimulated with 10 nM enalaprilat (Ept) or 10 nM desArg^{10}-kallidin (DAKD) in the absence or presence of Ca^{2+}-channel blocker Gd^{3+} (10 μM) or in calcium-free medium. A and B indicate control cell elevation of $[Ca^{2+}]_i$, whereas C and D show responses after 10-min preincubation with Gd^{3+}. E and F illustrate the results obtained with 10 nM Ept or 10 nM DAKD in calcium-free medium. Bars indicate 50 s. Absence of Ca^{2+} abolished the effect of enalaprilat, whereas desArg^{10}-kallidin gave a shorter response compared with that obtained in Ca^{2+}-containing medium. Arrows indicate the addition of ligands. Shown are representative tracings from 6 to 10 experiments.

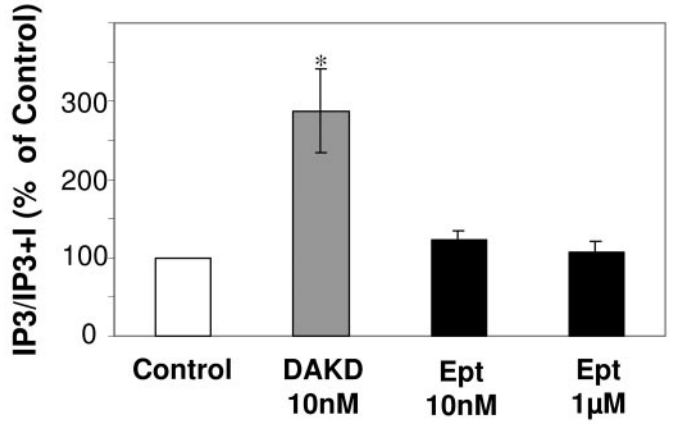

Fig. 4. [^{3}H]Inositol phosphate (IP) release in BPAE cells. BPAE cells were stimulated for 20 min with either desArg^{10}-kallidin (DAKD; 10 nM) or enalaprilat (Ept; 10 nM and 1 μM). In contrast to the peptide agonist desArg^{10}-kallidin, enalaprilat did not stimulate IP production in BPAE cells. Means ± S.E. (n = 5; done in duplicate). $\star$, $p < 0.05$, significantly different from control.

NO Release. Similar to the results with BPAE cells, both desArg^{10}-kallidin and enalaprilat caused prolonged NO release in stimulated human endothelial cells (Fig. 6A). DesArg^{10}-Leu^9-kallidin, a specific B_1 receptor antagonist (1 μM), blocked both of these responses (86 and 81% inhibition of desArg^{10}-kallidin and enalaprilat effects, respectively; data not shown), whereas HOE140, a B_2 receptor antagonist, was ineffective (2 and 6% inhibition of desArg^{10}-kallidin and enalaprilat, respectively; data not shown). In control HLMVE cells not stimulated with inflammatory cytokines, enalaprilat (100 nM) produced approximately a 4-fold smaller response (78 ± 9 nM NO, n = 7; data not shown) compared with that obtained with stimulated cells (Fig. 6). This is consistent with previous findings obtained with the peptide agonist desArg^{10}-kallidin in the control versus stimulated HLMVE cells (Sangsree et al., 2003).

In contrast to BPAE cells, pretreatment with iNOS inhibitor 1400W (2 μM for 10 min) inhibited the majority (65 or 57%) of the NO response elicited by desArg^{10}-kallidin or enalaprilat (Fig. 6B). The eNOS inhibitor (N^{ω}-nitro-L-arginine; 2 μM) was much less effective and inhibited only 20 or 10% of NO released by desArg^{10}-kallidin or enalaprilat, respectively. Therefore, the intracellular Ca^{2+} sequestering agent BAPTA (25 μM for 30 min) did not inhibit either the

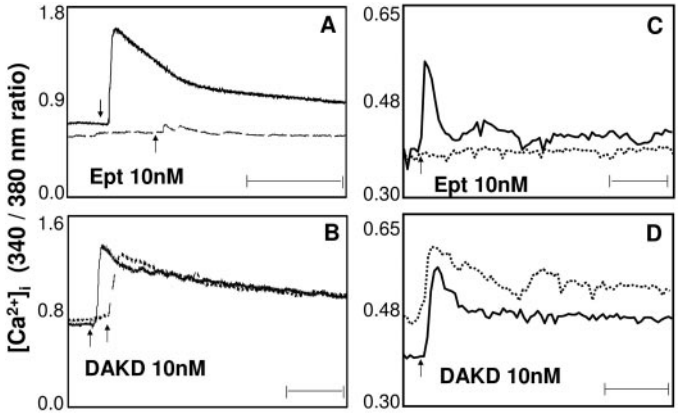

Fig. 5. Cholera toxin and calphostin C inhibit calcium increases stimulated by enalaprilat but not by desArg^{10}-kallidin. A and B, BPAE cells were preincubated with 1 μg/ml cholera toxin for 20 h and then stimulated with either 10 nM enalaprilat (Ept) or 10 nM desArg^{10}-kallidin (DAKD). Solid lines indicate the control responses, and broken lines represent results in cells preincubated with cholera toxin. Arrows, addition of ligands (Ept, DAKD). Bars, 100 s. Cholera toxin abolished only the enalaprilat effect, whereas the response to desArg^{10}-kallidin remained unchanged. Representative tracings from 4–7 experiments. C and D, BPAE cells were stimulated with either Ept (10 nM) or DAKD (10 nM) in the absence (solid lines) or presence (broken lines) of the protein kinase C inhibitor calphostin C (1 μM, 15-min preincubation). Bars, 100 s. Calphostin C selectively inhibited only the effect of enalaprilat, whereas the response to desArg^{10}-kallidin remained unchanged (representative tracings from six to seven experiments).

desArg^{10}-kallidin or enalaprilat effect (data not shown), which is in agreement with the fact that changes in $[Ca^{2+}]_i$ do not affect iNOS activity. Thus, in contrast to BPAE cells in which desArg^{10}-kallidin and enalaprilat activate NO synthesis by different mechanisms, in cytokine-stimulated HLMVE cells, the two agonists release NO via iNOS in a similar calcium-independent manner.

Arginine Uptake in HLMVE Cells. Because arginine uptake is an important regulator of iNOS, we tested whether desArg^{10}-kallidin and enalaprilat would enhance NOS activity by increasing arginine transport. Indeed, within 1 min, both desArg^{10}-kallidin and enalaprilat stimulated arginine uptake in HLMVE cells (Fig. 7). Lysine (10 mM), a competitive arginine-transport inhibitor, or desArg^{10}-Leu^{9}-kallidin (1 μM), a specific B_1 receptor blocker, abolished these effects (data not shown; $n = 2$). Positive results were obtained with 100 nM ramiprilat (data not shown; $n = 2$), but not with 100 nM lisinopril (data not shown; $n = 3$), which is consistent with previous data (Ignjatovic et al., 2002) showing that not all ACE inhibitors are agonists of the B_1 receptor.

In contrast to HLMVE cells, stimulation of B_1 receptors in BPAE cells by either desArg^{10}-kallidin or enalaprilat did not increase arginine-uptake (data not shown; $n = 2$, experiments done in triplicate).

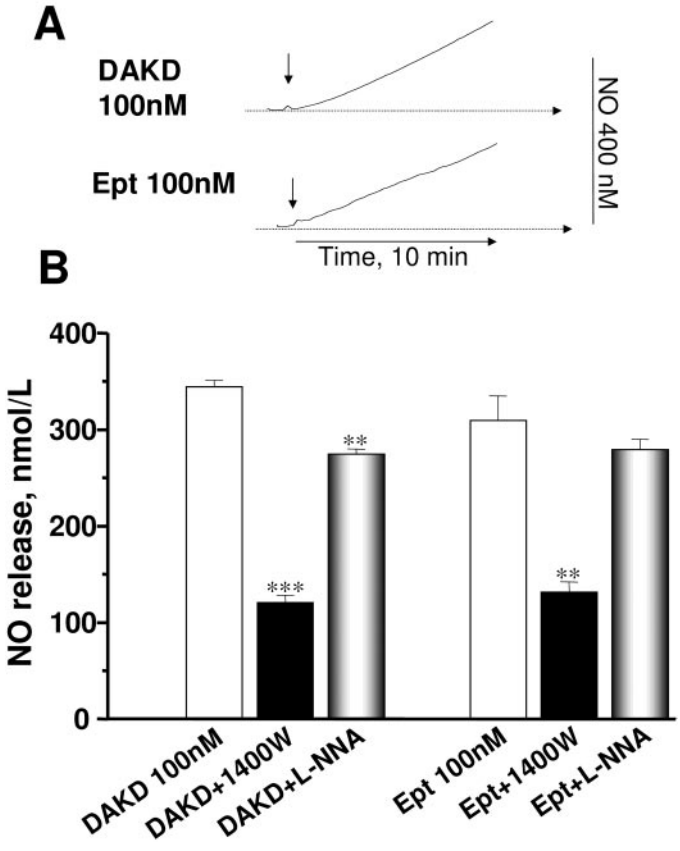

Fig. 6. DesArg^{10}-kallidin and enalaprilat release NO in stimulated HLMVE cells. Cells were stimulated with interleukin-1β and interferon-γ (see *Materials and Methods*) and then challenged with either 100 nM desArg^{10}-kallidin (DAKD) or 100 nM enalaprilat (Ept). A, typical real-time tracings. Vertical arrows indicate the addition of ligands, whereas horizontal ones indicate time in minutes. Vertical bar shows the response to standard 400 nM concentration of NO. B, mean values from four experiments (± S.E.) obtained with DAKD and Ept in the absence or presence of eNOS (2 μM L-NNA) or iNOS (2 μM 1400W) inhibitors. ⋆⋆, $p < 0.01$, significantly different from control; ⋆⋆⋆, $p < 0.001$, significantly different from control.

Discussion

The beneficial effects of ACE inhibitors in the treatment of various cardiovascular and renal conditions have been established in laboratory animals and in millions of patients worldwide. However, it is less known how they act on the cellular, subcellular, and molecular level. Using cultured cells, we found that ACE inhibitors in low nanomolar concentrations directly activate the B_1 receptor via the zinc-binding consensus sequence in the receptor (HEAWH) similar to those in the active sites of ACE (HEMGH) (Ignjatovic et al., 2002).

B_1 receptors seem to contribute to the effects of ACE inhibitors as tested in different systems, for example, when ACE inhibitors lowered blood pressure in laboratory animals (Marin-Castano et al., 2002). ACE inhibitors reduced extracellular signal-regulated kinase phosphorylation, matrix secretion, and mesangial cell proliferation associated with diabetic nephropathy, and B_1 receptor antagonist suppressed these effects (Mage et al., 2002). In addition, B_1 receptors are involved in the vasodilation caused by ACE inhibitor in patients with heart failure, but not in healthy individuals (Witherow et al., 2001), and even in the side effects (Hirata et al., 2003).

To study how ACE inhibitors can release NO by activating B_1 receptors, we used BPAE cells as a prototype for cells containing eNOS and B_1 receptor and then HLMVE cells, in which we induced B_1 and iNOS. We found that although the mode of NO release by ACE inhibitor and peptide ligand differs with bovine endothelial cells, it is quite similar with regard to the activation of B_1 receptor and iNOS in human cells treated with cytokines. The routinely used B_1 receptor blocker (desArg^{10}-Leu^{9}-kallidin) inhibited both peptide and ACE inhibitor agonists.

BPAE cells express significant levels of native B_1 receptors under basal conditions (Ignjatovic et al., 2002). Cytokines, which we used as inducers in HLMVE cells, did not significantly enhance B_1-mediated responses in BPAE cells (data not shown).

Stimulation of constitutively expressed B_1 receptors in

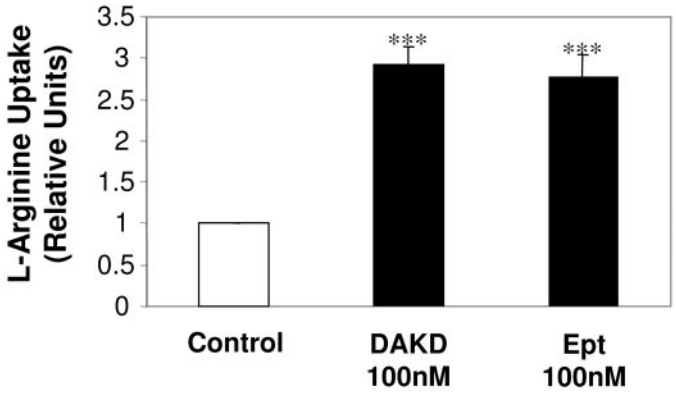

Fig. 7. DesArg^{10}-kallidin and enalaprilat increase L-arginine uptake in HLMVE cells. HLMVE cells were stimulated for 1 min with 100 nM desArg^{10}-kallidin (DAKD) or 100 nM enalaprilat (Ept). The abscissa shows the uptake of arginine relative to control. The results represent the means ± S.E. from nine independent experiments (performed in triplicate). ⋆⋆⋆, $p < 0.001$, significantly different from the control.

BPAE cells releases NO predominantly via eNOS activation and leads to a prolonged, high-output NO synthesis. This contrasts to the activation of the B_2 receptors by bradykinin, which brings a much shorter NO release (Ignjatovic et al., 2002). B_1 receptors generally produce prolonged responses as a consequence of limited desensitization and internalization (Mathis et al., 1996; Austin et al., 1997). On the other hand, B_1 receptor stimulation may activate eNOS by phosphorylation. This activation mechanism generally leads to a more prolonged, sustained NO output compared with the classic pathway mediated by elevated $[Ca^{2+}]_i$ (Skidgel, 2002). This may explain how enalaprilat released a reduced but still significant amount of NO, even in the presence of the intracellular calcium chelator BAPTA.

Although both desArg^{10}-kallidin and enalaprilat cause a sustained NO release from BPAE cells, the mechanism differs. The release of NO by desArg^{10}-kallidin is connected with the elevation of $[Ca^{2+}]_i$ and eNOS activation. Enalaprilat releases NO largely independent of $[Ca^{2+}]_i$, indicating that it should also involve additional mechanisms of eNOS activation (e.g., phosphorylation) or iNOS stimulation, as shown by partial inhibition with 1400W. Some iNOS can be present and functional in control unstimulated BPAE cells (Nelin et al., 2001), which is also indicated with the use of NOS inhibitors (Fig. 1).

Activation of bovine B_1 receptors stimulated sustained NO synthesis predominantly via eNOS activity without enhancing arginine uptake (not shown). This may be caused by a separate intracellular arginine pool (Closs et al., 2000), not freely exchangeable with arginine in the extracellular space and reported to be accessible to eNOS but not to iNOS (Closs et al., 2000).

B_1 receptor ligands desArg^{10}-kallidin and enalaprilat also increased intracellular calcium via distinct mechanisms in BPAE cells. ACE inhibitors trigger the influx of external calcium, independent of IP_3 generation, whereas desArg^{10}-kallidin first releases internal calcium via IP_3 liberation and then subsequently stimulates calcium entry. Therefore, when ACE inhibitors activate the B_1 receptors in BPAE cells, calcium enters from the medium, whereas desArg^{10}-kallidin produces a biphasic response. The initial sharp increase and decrease of $[Ca^{2+}]_i$ stimulated by desArg^{10}-kallidin probably represents G_q-mediated activation of phospholipase Cβ and the release of calcium from internal stores caused by the activation of IP_3 receptor in the endoplasmic reticulum. The later, sustained phase of elevated $[Ca^{2+}]_i$ is maintained by the entry of external calcium through a channel different from that blocked by Gd^{3+}, and therefore it is absent in calcium-free medium.

The mechanisms underlying receptor-activated Ca^{2+} influx into the cytosol are complex and not yet fully characterized. Receptor activation can open plasma membrane cation channels in various ways (Barritt, 1999). We attempted to characterize the Ca^{2+} entry channels involved by using the lanthanide Gd^{3+} because it competitively antagonizes Ca^{2+} (Erdös et al., 1960) and in low concentration specifically inhibits the store-operated, capacitance calcium channels (Putney et al., 2001). Indeed, Gd^{3+} abolished the increase of $[Ca^{2+}]_i$ elicited by enalaprilat without affecting the action of desArg^{10}-kallidin. Calcium entry stimulated by enalaprilat seems to depend on protein kinase C activation, because two different protein kinase C inhibitors blocked it. Protein kinase C can regulate several different members of transient receptor potential C and V subfamilies expressed in the endothelium (Nilius et al., 2003). Protein kinase C may modulate endothelial channels through phosphorylation of $G_{\beta\gamma}$ binding sites and subsequent antagonism of $G_{\beta\gamma}$-induced inhibition (Zamponi et al., 1997).

Different agonists acting on the same receptor type can cause differential coupling to G proteins leading to different effector pathways (Shraga-Levine and Sokolovsky, 2000). The selective blocking of enalaprilat's effect with cholera toxin suggests that desArg^{10}-kallidin and enalaprilat induce differential coupling of B_1 receptor to G proteins in bovine endothelial cells. The effects of enalaprilat may be mediated via G_s (cholera toxin-sensitive) and those of desArg^{10}-kallidin by a G_q pathway. B_1 receptor activation by peptide ligand is usually associated with $G_{\alpha q/11}$ and phospholipase Cβ activation (Austin et al., 1997), but a cholera toxin-sensitive, G_s pathway has also been reported (Christopher et al., 2001). We found enalaprilat to activate the B_1 receptor via a G_s-dependent pathway, but apparently it does so independent of protein kinase A activation, because its inhibitor was ineffective. This is consistent with reports on the existence of novel G_s signaling pathways independent of protein kinase A (Gu et al., 2000).

B_1 receptors are generally absent under physiological conditions in most cells but are rapidly induced in pathological settings. Inflammatory mediators, ischemia, atherosclerosis, tissue trauma, disruption of the B_2 receptor gene, or other pathological stimuli promote the expression of B_1 receptors (McLean et al., 2000; Duka et al., 2001). Acute coronary syndromes are accompanied by the cellular infiltration and release of cytokines, which are directly cytotoxic to cardiomyocytes (Force et al., 2004). Activation of induced B_1 receptors releases NO, and this may be advantageous under these conditions.

To simulate such a pathological milieu, we used human endothelial cells (HLMVE) and exposed them to cytokines, interleukin-1β, and interferon-γ for 16 to 18 h. Although the control HLMVE cells express low levels of B_1 receptors, the response to the peptide ligand desArg^{10}-kallidin is significantly up-regulated by cytokines (Sangsree et al., 2003). At the same time, iNOS and carboxypeptidase M (Sangsree et al., 2003), which removes the C-terminal arginine of kinins to convert them from B_2 to B_1 receptor agonists, are also induced. Carboxypeptidase-mediated arginine release can also stimulate NO production by iNOS in rat lungs and rat lung microvascular endothelial cells (Hadkar et al., 2004).

In contrast to BPAE cells, desArg^{10}-kallidin or ACE inhibitor activates B_1 receptors in cytokine-stimulated HLMVE cells to release NO predominantly via iNOS stimulation, as shown with NOS inhibitors (Fig. 6). This action results in prolonged NO release, mediated by enhanced arginine uptake and independent of $[Ca^{2+}]_i$ elevation. iNOS is primarily regulated at the transcriptional level; thus, the supply of extracellular arginine is necessary for this isoform. Indeed, in the absence of extracellular arginine uptake by system y+ or depletion of extracellular arginine, iNOS activity is abolished (Stevens et al., 1996; Closs et al., 2000). Because we found that B_1 receptor stimulation by ACE inhibitors or peptide ligand increased arginine uptake, this is a mode of enhancing iNOS activity in human endothelial cells. How B_1 receptors signal to activate downstream arginine transport is

still to be determined. Up-regulation and activation of iNOS can be detrimental in endothelial cells, but iNOS may also protect against oxidative damage after exposure to inflammatory cytokines (Hemmrich et al., 2003; Suschek et al., 2003).

In conclusion, these studies build on our previous report of a novel mode of action of ACE inhibitors to directly activate B_1 receptors independent of ACE and endogenous kinins (Ignjatovic et al., 2002). We described here how both types of B_1 receptor agonists, ACE inhibitors and a peptide ligand, stimulate eNOS and iNOS. ACE inhibitors enhance NO release from BPAE cells, which constitutively express B_1 receptors, differently than desArg^{10}-kallidin by releasing different second messengers. In contrast, desArg^{10}-kallidin and enalaprilat release NO by similar mechanisms independent of calcium in cytokine-stimulated HLMVE cells in which B_1 receptors were coinduced with iNOS. To the best of our knowledge, this is the first report that ACE inhibitors directly activate B_1 receptors to release NO in human endothelial cells.

References

Alderton WK, Cooper CE, and Knowles RG (2001) Nitric oxide synthases: structure, function and inhibition. *Biochem J* **357:**593–615.

Asano K, Chee CB, Gaston B, Lilly CM, Gerard C, Drazen JM, and Stamler JS (1994) Constitutive and inducible nitric oxide synthase gene expression, regulation and activity in human lung epithelial cells. *Proc Natl Acad Sci USA* **91:**10089–10093.

Austin CE, Faussner A, Robinson HE, Chakravarty S, Kyle DJ, Bathon JM, and Proud D (1997) Stable expression of the human kinin B1 receptor in Chinese hamster ovary cells. Characterization of ligand binding and effector pathways. *J Biol Chem* **272:**11420–11425.

Barritt GJ (1999) Receptor-activated Ca^{2+} inflow in animal cells: a variety of pathways tailored to meet different intracellular Ca^{2+} signalling requirements. *Biochem J* **337 (Pt 2):**153–169.

Carretero OA and Rhaleb NE (2001) Kinins and Ac-SDKP in the therapeutic effects of angiotensin-converting enzyme (ACE) inhibitors, in *Angiotensin-Converting Enzyme (ACE): Clinical and Experimental Insights* (Giles TD ed) pp 147–157, Health Care Communications, Inc., Fort Lee, NJ.

Christopher J, Velarde V, and Jaffa AA (2001) Induction of B_1-kinin receptors in vascular smooth muscle cells: cellular mechanisms of map kinase activation. *Hypertension* **38:**602–605.

Closs EI, Scheld JS, Sharafi M, and Forstermann U (2000) Substrate supply for nitric-oxide synthase in macrophages and endothelial cells: role of cationic amino acid transporters. *Mol Pharmacol* **57:**68–74.

Duka I, Kintsurashvili E, Gavras I, Johns C, Bresnahan M, and Gavras H (2001) Vasoactive potential of the B_1 bradykinin receptor in normotension and hypertension. *Circ Res* **88:**275–281.

Erdös EG, Debay CR, and Westerman MP (1960) Arylesterases in blood: effect of calcium and inhibitors. *Biochem Pharmacol* **5:**173–186.

Erdös EG, Deddish PA, and Marcic BM (1999) Potentiation of bradykinin actions by ACE inhibitors. *Trends Endocrinol Metab* **10:**223–229.

Erdös EG and Skidgel RA (1997) Metabolism of bradykinin by peptidases in health and disease, in *The Kinin System* (Farmer SG ed) pp 101–141, Academic Press, Inc., San Diego.

Fathy DB, Kyle DJ, and Leeb-Lundberg LM (2000) High-affinity binding of peptide agonists to the human B1 bradykinin receptor depends on interaction between the peptide N-terminal L-lysine and the fourth extracellular domain of the receptor. *Mol Pharmacol* **57:**171–179.

Ferro A, Queen LR, Priest RM, Xu B, Ritter JM, Poston L, and Ward JP (1999) Activation of nitric oxide synthase by beta 2-adrenoceptors in human umbilical vein endothelium in vitro. *Br J Pharmacol* **126:**1872–1880.

Force T, Kuida K, Namchuk M, Parang K, and Kyriakis JM (2004) Inhibitors of protein kinase signaling pathways: emerging therapies for cardiovascular disease. *Circulation* **109:**1196–1205.

Gu C, Ma YC, Benjamin J, Littman D, Chao MV, and Huang XY (2000) Apoptotic signaling through the β-adrenergic receptor. A new Gs effector pathway. *J Biol Chem* **275:**20726–20733.

Hadkar V, Sangsree S, Vogel SM, Brovkovych V, and Skidgel RA (2004) Carboxypeptidase-mediated enhancement of nitric oxide production in rat lungs and microvascular endothelial cells. *Am J Physiol* **287:**L35–L45.

Hemmrich K, Suschek CV, Lerzynski G, and Kolb-Bachofen V (2003) iNOS activity is essential for endothelial stress gene expression protecting against oxidative damage. *J Appl Physiol* **95:**1937–1946.

Hirata R, Nabe T, and Kohno S (2003) Augmentation of spontaneous cough by enalapril through up-regulation of bradykinin B_1 receptors in guinea pigs. *Eur J Pharmacol* **474:**255–260.

Ignjatovic T, Tan F, Brovkovych V, Skidgel RA, and Erdös EG (2002) Novel mode of action of angiotensin I converting enzyme inhibitors: direct activation of bradykinin B1 receptor. *J Biol Chem* **277:**16847–16852.

Kanno K, Hirata Y, Imai T, Iwashina M, and Marumo F (1994) Regulation of inducible nitric oxide synthase gene by interleukin-1 beta in rat vascular endothelial cells. *Am J Physiol* **267:**H2318–H2224.

Kolyada AY and Madias NE (2001) Transcriptional regulation of the human iNOS gene by IL-1beta in endothelial cells. *Mol Med* **7:**329–343.

Linz W, Wiemer G, Gohlke P, Unger T, and Scholkens BA (1995) Contribution of kinins to the cardiovascular actions of angiotensin-converting enzyme inhibitors. *Pharmacol Rev* **47:**25–49.

Mage M, Pecher C, Neau E, Cellier E, Dos Reiss ML, Schanstra JP, Couture R, Bascands JL, and Girolami JP (2002) Induction of B1 receptors in streptozotocin diabetic rats: possible involvement in the control of hyperglycemia-induced glomerular Erk 1 and 2 phosphorylation. *Can J Physiol Pharmacol* **80:**328–333.

Marcic B, Deddish PA, Skidgel RA, Erdös EG, Minshall RD, and Tan F (2000) Replacement of the transmembrane anchor in angiotensin I-converting enzyme (ACE) with a glycosylphosphatidylinositol tail affects activation of the B2 bradykinin receptor by ACE inhibitors. *J Biol Chem* **275:**16110–16118.

Marin-Castano ME, Schanstra JP, Neau E, Praddaude F, Pecher C, Ader JL, Girolami JP, and Bascands JL (2002) Induction of functional bradykinin B_1-receptors in normotensive rats and mice under chronic angiotensin-converting enzyme inhibitor treatment. *Circulation* **105:**627–632.

Mathis SA, Criscimagna NL, and Leeb-Lundberg LM (1996) B_1 and B_2 kinin receptors mediate distinct patterns of intracellular Ca^{2+} signaling in single cultured vascular smooth muscle cells. *Mol Pharmacol* **50:**128–139.

McLean PG, Perretti M, and Ahluwalia A (2000) Kinin B_1 receptors and the cardiovascular system: regulation of expression and function. *Cardiovasc Res* **48:**194–210.

Minshall RD, Tan F, Nakamura F, Rabito SF, Becker RP, Marcic B, and Erdös EG (1997) Potentiation of the actions of bradykinin by angiotensin I-converting enzyme inhibitors. The role of expressed human bradykinin B2 receptors and angiotensin I-converting enzyme in CHO cells. *Circ Res* **81:**848–856.

Nelin L, Nash HE, and Chicoine LG (2001) Cytokine treatment increases arginine metabolism and uptake in bovine pulmonary arterial endothelial cells. *Am J Physiol* **281:**L1232–L1239.

Nilius B, Droogmans G, and Wondergem R (2003) Transient receptor potential channels in endothelium: solving the calcium entry puzzle? *Endothelium* **10:**5–15.

Pfeffer M (2001) Angiotensin-converting enzyme (ACE) inhibitors in the prevention of cardiovascular disease: clinical evidence, in *Angiotensin-Converting Enzyme (ACE): Clinical and Experimental Insights* (Giles TD ed) pp 219–225, Health Care Communications, Inc., Fort Lee.

Putney JW Jr, Broad LM, Braun FJ, Lievremont JP, and Bird GS (2001) Mechanisms of capacitative calcium entry. *J Cell Sci* **114:**2223–2229.

Sangsree S, Brovkovych V, Minshall RD, and Skidgel RA (2003) Kininase I-type carboxypeptidases enhance nitric oxide production in endothelial cells by generating bradykinin B1 receptor agonists. *Am J Physiol* **284:**H1959–H1968.

Schanstra JP, Bataille E, Marin Castano ME, Barascud Y, Hirtz C, Pesquero JB, Pecher C, Gauthier F, Girolami JP, and Bascands JL (1998) The B1-agonist [des-Arg10]-kallidin activates transcription factor NF-kappaB and induces homologous upregulation of the bradykinin B1-receptor in cultured human lung fibroblasts. *J Clin Investig* **101:**2080–2091.

Seuwen K, Kahan C, Hartmann T, and Pouyssegur J (1990) Strong and persistent activation of inositol lipid breakdown induces early mitogenic events but not Go to S phase progression in hamster fibroblasts. Comparison of thrombin and carbachol action in cells expressing M1 muscarinic acetylcholine receptors. *J Biol Chem* **265:**22292–22299.

Shraga-Levine Z and Sokolovsky M (2000) Functional coupling of G proteins to endothelin receptors is ligand and receptor subtype specific. *Cell Mol Neurobiol* **20:**305–317.

Skidgel RA (2002) Proliferation of regulatory mechanisms for eNOS: an emerging role for the cytoskeleton. *Am J Physiol* **282:**L1179–L1182.

Stevens BR, Kakuda DK, Yu K, Waters M, Vo CB, and Raizada MK (1996) Induced nitric oxide synthesis is dependent on induced alternatively spliced CAT-2 encoding L-arginine transport in brain astrocytes. *J Biol Chem* **271:**24017–24022.

Suschek CV, Schnorr O, Hemmrich K, Aust O, Klotz LO, Sies H, and Kolb-Bachofen V (2003) Critical role of L-arginine in endothelial cell survival during oxidative stress. *Circulation* **107:**2607–2614.

Witherow FN, Helmy A, Webb DJ, Fox KA, and Newby DE (2001) Bradykinin contributes to the vasodilator effects of chronic angiotensin-converting enzyme inhibition in patients with heart failure. *Circulation* **104:**2177–2181.

Yang HY, Erdös EG, and Levin Y (1971) Characterization of a dipeptide hydrolase (kininase II: angiotensin I converting enzyme). *J Pharmacol Exp Ther* **177:**291–300.

Yang HYT and Erdös EG (1967) Second kininase in human blood plasma. *Nature (Lond)* **215:**1402–1403.

Zahler S, Kupatt C, and Becker BF (1999) ACE-inhibition attenuates cardiac cell damage and preserves release of NO in the postischemic heart. *Immunopharmacology* **44:**27–33.

Zamponi GW, Bourinet E, Nelson D, Nargeot J, and Snutch TP (1997) Crosstalk between G proteins and protein kinase C mediated by the calcium channel alpha1 subunit. *Nature (Lond)* **385:**442–446.

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