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Abstract—ACE inhibitors block B_2 receptor desensitization, thereby potentiating bradykinin beyond blocking its hydrolysis. Angiotensin (Ang)-(1-7) also acts as an ACE inhibitor and, in addition, may stimulate bradykinin release via angiotensin II type 2 receptors. In this study we compared the bradykinin-potentiating effects of Ang-(1-7), quinaprilat, and captopril. Porcine coronary arteries, obtained from 32 pigs, were mounted in organ baths, preconstricted with prostaglandin $F_{2\alpha}$, and exposed to quinaprilat, captopril, Ang-(1-7), and/or bradykinin. Bradykinin induced complete relaxation ($pEC_{50}=8.11\pm0.07$, mean$\pm$SEM), whereas quinaprilat, captopril, and Ang-(1-7) alone were without effect. Quinaprilat shifted the bradykinin curve to the left in a biphasic manner: a 5-fold shift at concentrations that specifically block the C-domain (0.1 to 1 nmol/L) and a 10-fold shift at concentrations that block both domains. Captopril and Ang-(1-7) monophasically shifted the bradykinin curve to the left, by a factor of 10 and 5, respectively. A 5-fold shift was also observed when Ang-(1-7) was combined with 0.1 nmol/L quinaprilat. Repeated exposure of porcine coronary arteries to 0.1 μmol/L bradykinin induced B_2 receptor desensitization. The addition of 10 μmol/L quinaprilat or Ang-(1-7) to the bath, at a time when bradykinin alone was no longer able to induce relaxation, fully restored the relaxant effects of bradykinin. Angiotensin II type 1 or 2 receptor blockade did not affect any of the observed effects of Ang-(1-7). In conclusion, Ang-(1-7), like quinaprilat and captopril, potentiates bradykinin by acting as an ACE inhibitor. Bradykinin potentiation is maximal when both the ACE C- and N-terminal domains are inhibited. The inhibitory effects of Ang-(1-7) are limited to the ACE C-domain, raising the possibility that Ang-(1-7) synergistically increases the blood pressure–lowering effects of N-domain–specific ACE inhibitors. (*Hypertension*. 2001;38:95-99.)

Key Words: angiotensin ■ bradykinin ■ angiotensin-converting enzyme inhibitors ■ receptors, bradykinin ■ coronary artery

Angiotensin (Ang)-(1-7) is a heptapeptide that is formed endogenously from both Ang I and Ang II.[1] In rats and dogs, Ang-(1-7) exerts direct vasodilatory effects via non–angiotensin II type 1 (AT_1), non–angiotensin II type 2 (AT_2) receptors, possibly by stimulating bradykinin and NO release.[1,2] In contrast, in humans or pigs, no direct vasodilatory effects of Ang-(1-7) were observed,[3–7] although Ang-(1-7) did antagonize the pressor effects of Ang II, suggesting that it may cause vasodilation indirectly, by acting as an AT_1 receptor antagonist.[5,6] In addition, Ang-(1-7) potentiates bradykinin, either via an AT_2 receptor–dependent mechanism or through inhibition of ACE.[3,5,8] The latter effect is not necessarily based on blockade of bradykinin hydrolysis, because recent studies have shown that ACE inhibitors, including Ang-(1-7), potentiate bradykinin by inhibiting desensitization of its receptor.[9–11] Somatic ACE has 2 homologous domains, each containing an active center. According to their position (N- or C-terminal), these domains are designated as the N- or C-domain, respectively. Interestingly, Ang-(1-7) inhibits the C-domain more potently than the N-domain (by 1 order of magnitude)[8] and is cleaved to Ang-(1-5) by the N-domain.[12]

In the present study, we investigated the bradykinin-potentiating effects of Ang-(1-7) in porcine coronary arteries (PCAs), its dependency on ACE, and the possible involvement of AT_1 and/or AT_2 receptors. The effects of Ang-(1-7) were compared with those of quinaprilat and captopril, 2 ACE inhibitors with preference for the ACE C-and N-domains, respectively.[13,14] We also verified the effect of Ang-(1-7) in human coronary arteries (HCAs).

Methods

Tissue Collection

HCAs were obtained from 4 "heart beating" organ donors (2 men and 2 women; age range, 14 to 38 years; mean$\pm$SEM age, 23 ± 5 years) who died of noncardiac causes (1 subarachnoid bleeding, 3 head trauma) <24 hours before the heart was taken to the laboratory. Hearts were provided by the Rotterdam Heart Valve Bank (Bio Implant Services/Eurotransplant Foundation, Rotterdam, The Netherlands) after removal of the aortic and pulmonary valves for transplantation purposes. The study was approved by the joint ethics committee of Erasmus University Rotterdam and University Hospital Rotterdam. Immediately after circulatory arrest, the hearts were stored in an ice-cooled, sterile, organ-protecting solution. After

arrival in the laboratory, the HCAs were removed and stored overnight in a cold, oxygenated Krebs' bicarbonate solution of the following composition (mmol/L): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, and glucose 8.3 (pH 7.4). Vessels were then cut into segments of $\approx$4 mm in length, suspended on stainless steel hooks in 15-mL organ baths containing Krebs' bicarbonate solution, aerated with 95% O_2/5% CO_2, and maintained at 37°C. Segments containing macroscopically visible atherosclerotic lesions were not used.

PCAs were obtained from 32 pigs age 2 to 3 months (Yorkshire×Landrace; weight, 10 to 15 kg). The pigs had been used in in vivo experiments studying the effects of α-adrenoceptor and serotonin receptor agonists and antagonists under pentobarbital (600 mg IV) anesthesia.[15] The ethics committee of Erasmus University Rotterdam dealing with the use of animals for scientific experiments approved the protocol for this investigation. Hearts were explanted at the end of the experiment, and the coronary arteries were removed immediately and handled in the same way as HCAs.

Organ Bath Studies

All vessel segments were allowed to equilibrate for at least 30 minutes, and the organ bath fluid was refreshed every 15 minutes during this period. Changes in tissue contractile force were recorded with a Harvard isometric transducer. The vessel segments, stretched to a stable force of $\approx$15 mN, were exposed to 30 mmol/L K^+ twice. The functional integrity of the endothelium was verified by observing relaxation to 1 nmol/L substance P after preconstriction with 1 μmol/L prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$).[16] Subsequently, the tissue was exposed to 100 mmol/L K^+ to determine the maximal contractile response to K^+. The segments were then allowed to equilibrate in fresh organ bath fluid for 30 minutes. Thereafter, the following experiments were performed.

First, Ang-(1-7) concentration-response curves (CRCs) (0.1 nmol/L to 10 μmol/L) were constructed in HCAs and PCAs, both at baseline and after preconstriction with 10 μmol/L $PGF_{2\alpha}$, in the absence or presence of the AT_1 receptor antagonist irbesartan (1 μmol/L) or the AT_2 receptor antagonist PD 123,319 (1 μmol/L).

Second, the effects of Ang-(1-7), quinaprilat, and captopril on bradykinin-induced vasorelaxation were studied in PCAs. Vessels were preincubated for 30 minutes in the absence or presence of Ang-(1-7) (10 pmol/L to 10 μmol/L), quinaprilat (0.1 pmol/L to 0.1 mmol/L), captopril (0.1 pmol/L to 0.1 mmol/L), 10 μmol/L Ang-(1-7)+1 μmol/L irbesartan, 10 μmol/L Ang-(1-7)+1 μmol/L PD 123,319, 10 μmol/L Ang-(1-7)+0.1 nmol/L quinaprilat, or 10 μmol/L Ang-(1-7)+10 μmol/L quinaprilat. Vessels were then preconstricted with 10 μmol/L $PGF_{2\alpha}$ or 1 μmol/L U46619, and CRCs to bradykinin (0.1 nmol/L to 1 μmol/L) were constructed.

Third, the effect of quinaprilat and Ang-(1-7) on desensitized B_2 receptors was studied in PCAs. Vessels were preincubated for 30 minutes with or without 1 μmol/L irbesartan or 1 μmol/L PD 123,319. Vessels were then preconstricted with 10 μmol/L $PGF_{2\alpha}$ and exposed 3 times to a concentration of bradykinin (0.1 μmol/L) that is capable of inducing maximal relaxation. Each next exposure was started as soon as the effect of the previous exposure had disappeared, ie, after $\approx$15 minutes. After the third exposure, when bradykinin no longer exerted a vasodilatory effect, quinaprilat (10 μmol/L) or Ang-(1-7) (10 μmol/L) was added to the organ bath. Thereafter, when the effects of quinaprilat and Ang-(1-7) had disappeared, a fourth bradykinin dose (0.1 μmol/L) was added to the organ bath.

Statistical Analysis

Data are given as mean±SEM and expressed as a percentage of the contraction in response to $PGF_{2\alpha}$ or U46619. CRCs were analyzed by the logistic function described by de Lean et al[17] to obtain pEC_{50} ($-{}^{10}$log EC_{50}) values, EC_{50} representing the concentration at which 50% of the maximal relaxant effect has been reached. Statistical analysis was by ANOVA, followed by post hoc evaluation (according to Tukey or Dunnett where appropriate). Values of $P<0.05$ were considered significant.
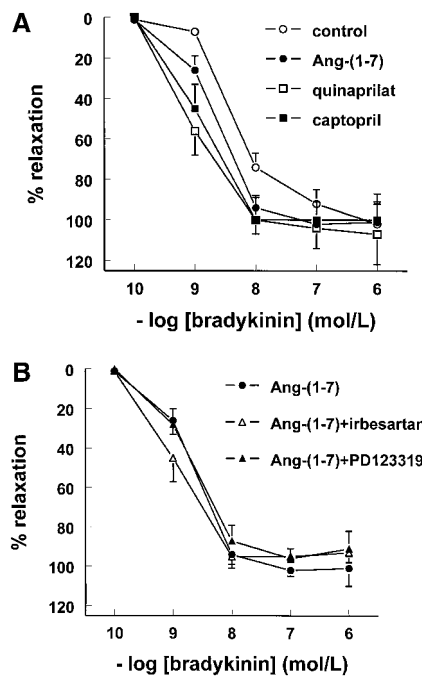

Figure 1. Relaxations of PCAs, preconstricted with 10 μmol/L $PGF_{2\alpha}$ or 1 μmol/L U46619, to bradykinin in the absence (control) or presence of 10 μmol/L Ang-(1-7), 10 μmol/L quinaprilat, or 10 μmol/L captopril (top). The effect of Ang-(1-7) on bradykinin-induced relaxations was also studied in the presence of 1 μmol/L irbesartan or 1 μmol/L PD 123,319 (bottom). Data (mean±SEM of 5 to 12 experiments) are expressed as a percentage of the contraction induced by $PGF_{2\alpha}$ or U46619. Ang-(1-7), quinaprilat, and captopril significantly shifted the bradykinin CRC to the left ($P<0.05$ vs control for Ang-(1-7); $P<0.01$ vs control for the 2 ACE inhibitors), and the effect of Ang-(1-7) was not influenced ($P=$NS) by the presence of irbesartan or PD 123,319.

Results

Effect of Ang-(1-7) on HCAs and PCAs

Ang-(1-7), at concentrations up to 10 μmol/L, did not exert a response in HCAs (n=4) and PCAs (n=5 to 12) at baseline or after preconstriction with $PGF_{2\alpha}$ or in the presence of irbesartan or PD 123,319.

Effects of Ang-(1-7), Quinaprilat, and Captopril on Bradykinin-Induced Relaxation in PCAs

Bradykinin caused complete relaxation of preconstricted PCAs in a concentration dependent–manner ($pEC_{50}=8.11\pm0.07$; n=12; Figure 1, top panel). Quinaprilat (10 μmol/L) and captopril (10 μmol/L), like Ang-(1-7) at this concentration, did not exert any effect on preconstricted PCAs. However, in the presence of 10 μmol/L Ang-(1-7), the bradykinin CRC was shifted $\approx$5-fold to the left ($pEC_{50}=8.72\pm0.09$; n=12; $P<0.05$ versus control), whereas in the presence of 10 μmol/L quinaprilat or 10 μmol/L captopril the bradykinin CRC was shifted $\approx$10-fold to the left ($pEC_{50}=9.03\pm0.21$ and 8.91 ± 0.09, respectively; n=8; $P<0.01$ versus control) (Figure 1, top panel). The effect of Ang-(1-7) on the bradykinin CRC was not affected by irbesartan or PD 123,319 (Figure 1, bottom panel).

To study the concentration dependency of the Ang-(1-7)– and ACE-inhibitor–induced leftward shifts, bradykinin CRCs
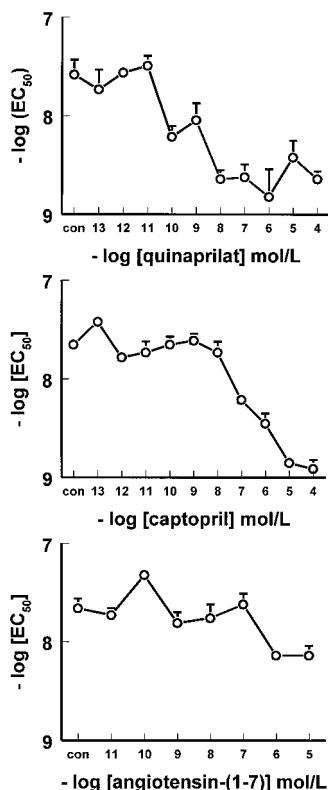
Figure 3. Relaxations of PCAs, preconstricted with 10 μmol/L PGF$_{2\alpha}$ or 1 μmol/L U46619, to bradykinin in the absence (control, + symbols) or presence of 0.1 nmol/L quinaprilat (squares) or 10 μmol/L quinaprilat (circles), with (closed symbols) or without (open symbols) 10 μmol/L Ang-(1-7). Data (mean±SEM of 11 to 13 experiments) are expressed as a percentage of the contraction induced by PGF$_{2\alpha}$ or U46619. The addition of Ang-(1-7) on top of quinaprilat did not have a significant effect.

Figure 2. Change in $-\log(EC_{50})$ of the bradykinin CRC in the presence of increasing concentrations of quinaprilat (top), captopril (middle), or Ang-(1-7) (bottom). An increase in $-\log(EC_{50})$ represents a leftward shift of the bradykinin CRC. Data (mean±SEM of 6 to 8 experiments) were obtained in PCAs preconstricted with 1 μmol/L U46619. Con indicates control. Significant differences ($P<0.05$) vs control were obtained for quinaprilat, captopril, and Ang-(1-7) at concentrations ≥ 0.1, 100, and 1000 nmol/L, respectively.

were also constructed in the presence of a wide range of Ang-(1-7), quinaprilat, and captopril concentrations (Figure 2). The leftward shift caused by quinaprilat occurred in a biphasic manner, with a 5-fold shift at concentrations in the subnanomolar range and a 10-fold shift at concentrations >1 nmol/L. This biphasic shift is in agreement with earlier studies[13] demonstrating different quinaprilat binding affinities of the ACE C- ($K_d=7$ pmol/L) and N-domains ($K_d=1267$ pmol/L) and suggests that the 5- and 10-fold shifts represent C-domain inhibition and complete (ie, C- plus N-domain) ACE inhibition, respectively. The 10-fold leftward shift caused by captopril occurred monophasically (Figure 2), suggesting that captopril is bound with similar affinity by the 2 ACE domains. The 5-fold leftward shift caused by Ang-(1-7) also occurred monophasically (Figure 2), suggesting either that Ang-(1-7) inhibits 1 ACE domain only or that Ang-(1-7) potentiates bradykinin independently of its effects on ACE. To sort out the latter, the effect of 10 μmol/L Ang-(1-7) on top of quinaprilat, either at a concentration that selectively inhibits the ACE C-domain (0.1 nmol/L) or at a concentration that inhibits both ACE domains (10 μmol/L), was studied. Ang-(1-7) affected neither the 5-fold leftward shift of the bradykinin CRC at 0.1 nmol/L quinaprilat ($pEC_{50}=8.59\pm0.39$ and 8.68 ± 0.29 with and without Ang-
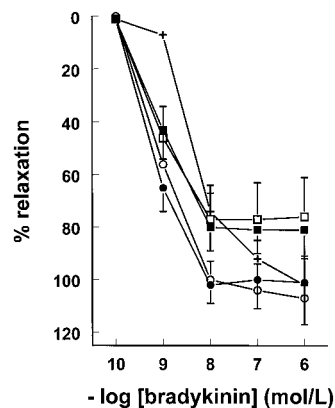
(1-7), respectively; n=13; $P=$NS) nor the 10-fold shift at 10 μmol/L quinaprilat ($pEC_{50}=9.06\pm0.20$ and 9.03 ± 0.21 with and without Ang-(1-7), respectively; n=11; $P=$NS) (Figure 3). Most likely, therefore, Ang-(1-7), at concentrations up to 10 μmol/L, acts as a selective inhibitor of the ACE C-domain.

Effect of Ang-(1-7) and Quinaprilat on Desensitized B$_2$ Receptors in PCAs

Repeated exposure of preconstricted vessel segments to 0.1 μmol/L bradykinin produced progressively smaller responses (Figure 4, top panel). The response to the third bradykinin dose was <50% of the response to the first bradykinin dose. Quinaprilat or Ang-(1-7), added to the organ bath after the effect of the third bradykinin dose had disappeared, both at a concentration of 10 μmol/L, completely restored the relaxant effect of bradykinin (90±10% and 83±8% relaxation, respectively; n=8; $P=$NS). A fourth bradykinin dose, added after the effect of quinaprilat or Ang-(1-7) had disappeared, induced no further effect. The effect of Ang-(1-7) was not different in the presence of irbesartan or PD 123,319 (Figure 4, bottom panel).

Discussion

The present study shows that Ang-(1-7) potentiates the vasodilator effects of bradykinin in PCAs through inhibition of the ACE C-domain. AT$_1$ or AT$_2$ receptors are not involved in this effect, nor did Ang-(1-7) exert direct (ie, independently of bradykinin) relaxant effects in either HCAs or PCAs. Furthermore, the maximal potentiating effect of Ang-(1-7) was smaller than that of the ACE inhibitors quinaprilat and captopril, suggesting that full potentiation will only be obtained when both ACE domains are inhibited.

The concentrations of Ang-(1-7) required to obtain ACE C-domain inhibition (≥ 1 μmol/L) are in agreement with previously reported values.[5,8] These concentrations exceed the in vivo tissue and plasma Ang-(1-7) concentrations in rats and humans by at least 4 orders of magnitude. Ang-(1-7)
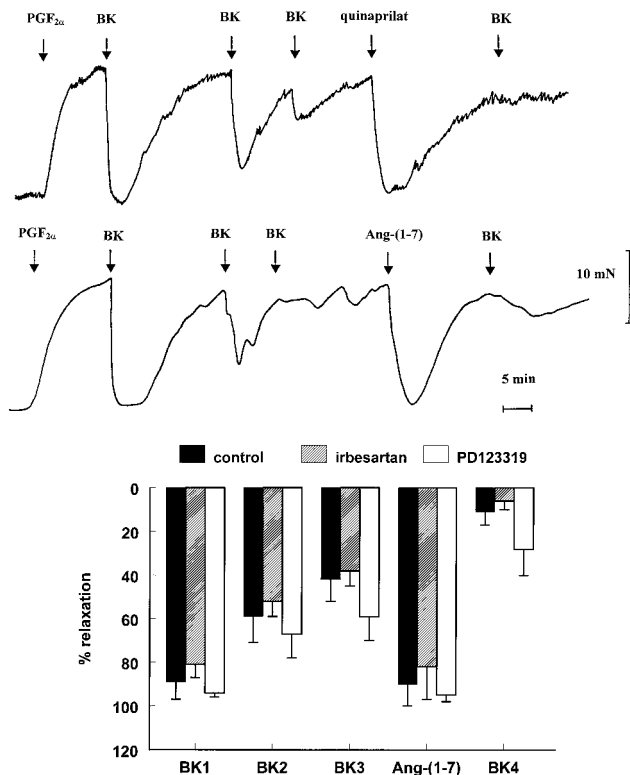
Figure 4. Top, Original tracings of an experiment in which a PCA, after preconstriction with 10 μmol/L PGF$_{2\alpha}$, is subsequently exposed to 0.1 μmol/L bradykinin (BK) (3 times), 10 μmol/L quinaprilat (top), or 10 μmol/L Ang-(1-7) (bottom), and 0.1 μmol/L bradykinin. Bottom, Relaxations of PCAs, after preconstriction with 10 μmol/L PGF$_{2\alpha}$, to 3 consecutive bradykinin doses (0.1 μmol/L; BK1, BK2, BK3), 10 μmol/L Ang-(1-7), and a fourth bradykinin dose (0.1 μmol/L; BK4) in the absence or presence of 1 μmol/L irbesartan or 1 μmol/L PD 123,319. Data (mean±SEM of 4 to 8 experiments) are expressed as a percentage of the contraction induced by PGF$_{2\alpha}$.

levels increase during ACE inhibition[18–21] because ACE inhibitors block the ACE N-domain–mediated degradation of Ang-(1-7).[1,8] However, the fact that most ACE inhibitors also block the ACE C-domain will mask any potential C-domain–blocking effect of the high Ang-(1-7) levels during ACE inhibition. Indeed, Ang-(1-7) did not enhance bradykinin-induced vasodilation in the forearm in human subjects treated with an ACE inhibitor.[7] Therefore, the effects of Ang-(1-7) described in the present study may only be of physiological importance if sufficiently high concentrations of Ang-(1-7) are reached in the immediate vicinity of ACE (the levels measured per gram tissue do not exclude this possibility) and/or in combination with ACE inhibitors that specifically block the ACE N-domain.

Studies with the N-domain–specific substrate *N*-acetyl-ser-asp-lys-pro (AcSDKP) have revealed that the ACE inhibitor captopril inhibits AcSDKP hydrolysis 16 times more potently than Ang I hydrolysis.[14] Because the ACE N- and C-domains exhibit similar catalytic activities toward Ang I,[22] these data suggest that captopril, at low concentrations, preferentially inhibits the ACE N-domain. Using captopril, however, we were unable to make a clear distinction between the effects of ACE N- and C-domain inhibition on the relaxant effects of

bradykinin. In contrast, using the ACE inhibitor quinaprilat, which binds to the ACE C-domain with almost 200-fold greater affinity than to the ACE N-domain,[13] we could make such a distinction: quinaprilat shifted the bradykinin CRC to the left in a biphasic manner, with an ≈5-fold shift occurring at concentrations that selectively block the C-domain and an ≈10-fold shift at concentrations that block both domains. Our inability to find a similar biphasic shift in the presence of increasing concentrations of captopril most likely relates to the limited selectivity of this ACE inhibitor for the ACE N-domain.

Ang-(1-7), like captopril, induced a monophasic leftward shift of the bradykinin CRC. At the highest Ang-(1-7) concentration (10 μmol/L) that was tested, however, the shift was only half of that caused by similar concentrations of quinaprilat or captopril. Adding quinaprilat, at a concentration that selectively blocks the ACE C-domain, to 10 μmol/L Ang-(1-7) did not cause a further leftward shift, thereby demonstrating that Ang-(1-7), at least at this concentration, blocks the ACE C-domain only. Because a concentration of 10 μmol/L is already many orders of magnitude above the measured concentrations of Ang-(1-7) in vivo,[18–21] we did not evaluate whether even higher concentrations of Ang-(1-7) resulted in ACE N-domain inhibition.

Does ACE inhibition result in bradykinin potentiation by blocking its hydrolysis? Because bradykinin, like Ang I, is cleaved equally well by the 2 ACE domains,[22] our finding that blockade of both domains results in a twice as large leftward shift as blockade of 1 domain appears to support this possibility. However, the long half-life of bradykinin in this preparation (30 minutes),[3] as well as our previous finding in PCAs that the quinaprilat-induced leftward shift of the ACE-resistant bradykinin analogue [Hyp^3-Tyr(Me)^8]-bradykinin was not different from that of bradykinin,[11] argues against this concept. Moreover, under conditions of B$_2$ receptor desensitization (induced by repeated exposure to a concentration of bradykinin that causes maximal relaxation), when bradykinin itself was no longer active, both quinaprilat and Ang-(1-7) immediately restored the effect of bradykinin. Taken together, therefore, a more likely explanation for the Ang-(1-7)–, quinaprilat-, and captopril-induced bradykinin potentiation is the upregulation (or resensitization) of B$_2$ receptors that has recently been described in Chinese hamster ovary (CHO) cells transfected with the human B$_2$ receptor and human ACE.[8,9,23,24] This effect only occurs when ACE and B$_2$ receptors are sterically closely associated, probably forming a heterodimer.[24] ACE inhibitors are believed to alter the heterodimer interaction, thereby promoting a conformation in the B$_2$ receptor that affects its sequestration and coupling to second messengers.[24] Our findings in intact PCAs show that ACE-B$_2$ receptor crosstalk is not limited to transfected CHO cells and, in addition, suggest that the conformational changes underlying bradykinin potentiation correlate directly with inhibition of the ACE C- and N-domains.

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