Heterodimerization of Type A and B Cholecystokinin Receptors Enhance Signaling and Promote Cell Growth*

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Dimerization of several G protein-coupled receptors has recently been described, but little is known about its clinical and functional relevance. Cholecystokinin (CCK) and gastrin are structurally related gastrointestinal and neuronal peptides whose functions are mediated by two structurally related receptors in this superfamily, the type A and B CCK receptors. We previously demonstrated spontaneous homodimerization of type A CCK receptors and the dissociation of those complexes by agonist occupation (Cheng, Z. J., and Miller, L. J. (2001) J. Biol. Chem. 276, 48040–48047). Here, for the first time, we also demonstrate spontaneous homodimerization of type B CCK receptors, as well as heterodimerization of that receptor with the type A CCK receptor. Unlike type A CCK receptor dimers, the homodimerization of type B CCK receptors was not affected by ligand occupation. However, although heterodimers of type A and B CCK receptors bound natural agonists normally, they exhibited unusual functional and regulatory characteristics. Such complexes demonstrated enhanced agonist-stimulated cellular signaling and delayed agonist-induced receptor internalization. As a likely consequence, agonist-stimulated cell growth was markedly enhanced in cells simultaneously expressing both of these receptors. Our results provide the first evidence that heterodimerization of G protein-coupled receptors can form a more “powerful” signaling unit, which has potential clinical significance in promoting cell growth.

G protein-coupled receptors represent the largest and most diverse superfamily of transmembrane receptors and mediate the effects of a wide variety of extracellular stimuli. It is now well established that, like single transmembrane tyrosine kinase receptors (2), these heptahelical receptors can form oligomeric complexes in the plasma membrane (3–13). Evidence for this includes biochemical, biophysical, and functional data (3, 6, 7). In addition to homodimerization, heterodimerization can occur with some structurally related receptors in this superfamily (8–10, 12). This has been implicated in the effects on ligand binding and acute effects on cellular signaling (8, 9, 12). Formation of heterodimers of nonfunctional B1 and B2 γ-amino-nobutyric acid receptors have been shown to be necessary for binding and complete functional activity of native ligands (10). Dimeric complexes of fully functional opioid receptors have been shown to alter their selectivity, displaying decreased affinities for highly selective agonists and enhanced affinities for nonselective agonists (9). Dimerization between somatostatin receptors has also been found to alter the pharmacology and signaling of individual receptors (12). However, little is known about the relevance of receptor dimerization to longer term effects, such as cell growth.

Gastrin and cholecystokinin (CCK)3 are gastrointestinal and neuronal peptides with important regulatory roles in the digestive tract and nervous system, including both acute and more chronic trophic effects (14–16). These functions are mediated by two receptors, the type A and type B CCK receptors, both belonging to the class I family of G protein-coupled receptors and exhibiting 48% structural homology with each other (17, 18). These two receptors can be distinguished on the basis of their structural specificity, with both recognizing gastrin and CCK but with each having markedly distinct sensitivities to the state of tyrosine sulfation of those peptides.

Although additional subtypes of CCK receptors have been predicted to exist, based on the complexity of the pharmacology and physiology of gastrin and CCK in vivo, only these two CCK receptor cDNAs have thus far been identified. It is possible that receptor oligomerization contributes to the pharmacology that has been observed and may even play a role in cell growth. We recently utilized bioluminescence resonance energy transfer (BRET) and immunoprecipitation to demonstrate that type A CCK receptors can exist as homodimers in living cells, with these complexes dissociated by agonist binding (1). To date there have been no reports of the ability of type B CCK receptors to dimerize or the possibility of the type A and B CCK receptors to form heterodimeric complexes.

Indeed, using the same biophysical techniques, our current study demonstrated homodimerization of type B CCK receptors, as well as heterodimerization of this receptor with type A CCK receptors when co-expressed in cells. Of note, unlike the type A receptors (1), oligomerization of the type B CCK receptor was not affected by agonist binding. Further functional studies showed that heterodimers of CCK receptors display new functional and regulatory properties affecting nonpeptidyl ligand binding and agonist-induced calcium signaling and receptor trafficking that were distinct from those of individual receptors. Of note, we found that heterodimer-expressing cells displayed significantly increased agonist-stimulated cell

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† The abbreviations used are: CCK, cholecystokinin; G-17, gastrin-17; BRET, bioluminescence resonance energy transfer; Rlu, Renilla luciferase; YFP, yellow fluorescent protein; HA, hemagglutinin; CHO, Chinese hamster ovary; HPLC, high pressure liquid chromatography; PBS, phosphate-buffered saline; FITC, fluorescein isothiocyanate.
growth. These results support the formation of a novel receptor signaling unit by dimerization of G protein-coupled receptors and has potentially important functional implications.

**EXPERIMENTAL PROCEDURES**

**cDNA Constructs for Receptor Expression Studies**—The human type A and type B CCK receptor cDNA constructs were prepared as we have described (17, 19). A series of epitope-tagged forms of these receptors were prepared. Type B CCK receptor constructs having an influenza hemagglutinin (HA) epitope tag at the amino terminus and type A CCK receptor constructs having a FLAG epitope tag at its amino terminus were prepared using the QuickChange™ site-directed mutagenesis kit (Stratagene). The receptor constructs were verified by sequencing and confirmed by Western blotting with an anti-FLAG antibody (Sigma) in PBS at 4°C with the appropriate concentration of ligand. The peak intracellular calcium transients were utilized to determine the concentration dependence of the biological responses.

**Bioluminescence and Fluorescence Assays**—Bioluminescence and fluorescence measurements were performed with approximately one million transfected COS cells in a 1-ml cuvette using a SPEX Fluorolog III spectrofluorometer (SPEX Industries, Edison, NJ). The bioluminescence assay, the cell-permeant substrate of Renilla luciferase, coelenterazine (Packard Bioscience), was added to the cell suspension to yield an excitation of 5 μM. The bioluminescence emission was immediately monitored in the spectrum between 400 and 600 nm. To assay YFP fluorescence, the cells were excited at 490 nm, and emission was scanned from 500 to 580 nm.

**Cell Proliferation Assays**—The cells expressing type A and/or type B CCK receptors were lysed using Lipoprep (Invitrogen), respectively. The amplified products were ligated in frame into XhoI/XbaI sites of the receptor-bearing constructs. All of the sequences were verified by direct DNA sequencing. For controls, β2-adrenergic receptor constructs (β2-adrenergic receptor-Ru and β2-adrenergic receptor-YFP) were generously provided by Dr. Michel Bouvier (11).

**Transferrin Receptor Expression Systems**—Transient receptor expression was achieved in COS-1 cells. These were plated in 100-mm tissue culture plates at 0.75x10⁶ cells/plate in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 5% Fetal Clone II (HyClone) 20 h before transfection. Transfection was performed using the DEAE-dextran method with 3 μg of cDNA of each construct/plate (17). The levels of CCK receptor expression were monitored by radioligand binding assay and Western blot analysis.

**Confocal Fluorescence Microscopy**—Cellular calcium responses to CCK or gastrin were determined in receptor-bearing CHO cells using a well-characterized assay (1, 22).

**Receptor Trafficking Studies**—Confocal fluorescence microscopic analysis was performed on a Zeiss LSM510 microscope using a Zeiss 63x/1.4 numerical aperture oil immersion lens. For single labeling experiments, cells expressing HA-tagged type B CCK receptors and/or FLAG-tagged type A CCK receptors were lysed using Lipoprep in 24-well plates. They were washed twice with cold PBS and were labeled with 1 nm Rho-nsCCK for 1 h at 4°C. The coverslips were rinsed and incubated in PBS at 37°C for the time points indicated, followed by 2% paraformaldehyde fixation. The coverslips were then washed and mounted on slides. The subcellular distribution of type B CCK receptors was determined single-line excitation at 568 nm. In all experiments, the cells on coverslips were labeled with 1 nm Rho-nsCCK and anti-FLAG antibody (Sigma) in PBS at 4°C for 1 h. After rinsing, the coverslips were incubated in PBS at 37°C for 0 or 30 min, followed by incubation with goat anti-mouse FITC-conjugated secondary antibody in PBS with 0.02% saponin and 10% goat serum at room temperature for 1 h. The coverslips were then rinsed, and fluorescence images were acquired. The confocal images were overlaid with merge images with red and green fluorescence. The coverslips were then scanned and scanned at 515–540 nm for FITC, 568 nm for rhodamine and 590–610 nm for rhodamine. The coverslips were scanned at 515–540 nm for FITC, 568 nm for rhodamine and 590–610 nm for rhodamine.
Aqueous One Solution Cell Proliferation Assay (Promega) was used for the comparison of independent samples.

CCK receptors were plated at a density of 20,000 cells/well into 96-well dishes in Ham's F-12 with 10% Fetal Clone II and allowed to attach overnight. The cells were treated with varied concentrations of gastrin or CCK for 6 days, or with 10^{-7} M gastrin or CCK for times indicated in serum-free medium. The medium was changed every other day, and the cells were incubated in a CO_2 incubator at 37 °C. The CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega) was used according to the manufacturer's recommendations. The cells were incubated with the CellTiter reagent for 2 h, and the plates were then read at 490 nm using a 96-well plate reader. We performed standard curves in each cell line to show that there were linear responses between cell number and absorbance at 490 nm. The incubation of culture medium with the CellTiter reagent was used as background absorbance.

Statistical Analysis—The data were analyzed using Student's *t* test for comparison of independent samples.

**RESULTS**

**Preparation of Cells Expressing Tagged Receptor Constructs and Type B CCK Receptor Probe**—Each of the CCK receptor constructs incorporating epitope tags was prepared as described under “Experimental Procedures.” Each of them was synthesized in the cell and transported normally to the cell surface. Fig. 1 shows representative Western blots from cells expressing the receptor constructs individually or together, using anti-HA antibody or anti-FLAG antibody, respectively. As expected by the strategies we used to establish the stable cell lines, the cells transfected with both receptor cDNA constructs expressed levels of type B CCK receptors similar to that transfected with type B CCK receptor cDNA construct alone and levels of type A CCK receptors similar to that transfected with type A CCK receptor cDNA construct alone.

A new fluorescent nonsulfated CCK analogue (Rho-nsCCK) was developed and characterized to track type B CCK receptors. It bound to the type B CCK receptor, specifically and saturably with high affinity similar to that for wild type CCK receptors (data not shown). This provided adequate support to proceed with the establishment of stable cell lines expressing these constructs in CHO cells. The densities of receptors on the cell surface in the stable cell lines are shown in Table I. The cell lines utilized in the comparisons had numbers of type A and type B CCK receptors that were within 1.8-fold of each other. Both CCK and gastrin were used to compete with the CCK-like radioligand, 125I-[D-Ala^2,31]CCK-26-33 (nonselective ligand that binds with high affinity to both type A and type B CCK receptors) in membranes from the cells expressing type B, type A, or both types of CCK receptors (Table I). As previously reported, type B CCK receptors have high affinities for both CCK and gastrin, whereas type A CCK receptors have a high affinity for CCK and a low affinity for gastrin. The nonpeptidyl antagonists L-364,718 and L-365,260 were also utilized in competition binding assays, utilizing the same radioligand (Table I).

In contrast, cells expressing both type A and type B CCK receptors (type B-type A CCK receptor heterodimers) acted more like type B CCK receptors in their agonist binding properties, with high affinities for both agonists (Table I). In control experiments, the 1:1 mixture of membranes from two different cell lines individually expressing type A and type B CCK receptors were tested in the binding assay, and the binding affinities were compared with those of membranes from cells simultaneously expressing both receptors. Although its affinity for CCK was similar to that of heterodimers, its affinity for gastrin was clearly distinguishable from that of these heterodimers (IC_{50} value for heterodimers was 20 nM, whereas that for 1:1 mixture of membranes from type B and type A CCK receptor-expressing cells was 3 nM). Of particular interest, the type B-type A CCK receptor heterodimers exhibited decreased affinity for the nonpeptidyl ligands representing the type A CCK receptor antagonist L-364,718 and the type B CCK receptor antagonist L-365,260 (Table I). L-364,718 had a 100-fold lower affinity for the heterodimer complex than for type B CCK receptors, whereas L-365,260 had a 5-fold lower affinity for the heterodimer complex than for type B CCK receptors. Thus, the formation of complexes was able to nullify the high affinity benzodiazepine binding activities of the individual receptors.

**Biological Characterization of CCK Receptor-bearing Cells**—CCK elicited similar intracellular calcium responses at all epitope-tagged receptor constructs compared with the analogous wild type receptor construct expressed in the same type of cell (Table II). No significant differences were observed for the maximal intracellular calcium responses between the tagged CCK receptor constructs and wild type CCK receptor (*p* < 0.05).

We also examined the effects of heterodimerization of type A and type B CCK receptors on the agonist-stimulated intracellular calcium responses. As expected, CCK elicited strong calcium responses in cells expressing each receptor subtype, whereas gastrin elicited a strong calcium response in cells...
expressing type B but not type A CCK receptors only. Of note, the activation of type B-type A CCK receptor heterodimers by CCK as well as gastrin resulted in significant increases in calcium responses, compared with those in the cells expressing only type B CCK receptors (Fig. 2). These calcium responses in the heterodimer-expressing CHO cells were much greater than that from the mixture (1:1) of cells expressing each receptor alone (data not shown). Consistent with the reduced ability of the heterodimer-expressing cells to bind nonpeptidyl antagonist ligands, the CCK-stimulated signaling response in these cells was only minimally blocked by L-364,718, whereas a high concentration of L-365,260 modestly inhibited the gastrin-induced biological activity (data not shown).

Significant BRET signals were detected for the cells expressing two tagged type A CCK receptors, two tagged type B CCK receptors, or the combination of both tagged type A and B CCK receptors, indicating the homodimerization and heterodimerization of CCK receptors. Co-expression of either wild type A or B CCK receptors could specifically inhibit BRET signals from complexes incorporating those receptors, whereas co-expression of the β2-adrenergic receptor did not have any effect. Similarly, BRET between adrenergic receptors was unaffected by expression of CCK receptors. Because we have carefully monitored the expression levels of all of the receptor constructs and kept them at constant levels, these results suggest that the BRET signals detected are due to specific interaction between receptors.

**Effect of Ligand Occupation on Receptor BRET**—Agonist occupation of receptors has been variably reported to increase (24), to decrease (7, 25), or to have no effect (26) on the dimerization of GPCRs. In the case of type A CCK receptors, we have reported that agonist could dissociate the constitutive oligomeric receptors. In contrast, however, agonist treatment (either CCK or gastrin) had no effect on either homodimers or heterodimers that included type B CCK receptors (Table III).

**Agonist-induced Receptor Internalization**—Previously, we showed that type A CCK receptors undergo rapid agonist-induced internalization (27). Here, the redistribution of type B CCK receptors upon agonist occupation was visualized using the type B CCK receptor-specific agonist Rho-nsGCck (Fig. 5), and the internalization of type B CCK receptors was quantified by Metamorph software (Fig. 5). Indeed, as shown in the time course curves of cells expressing type B CCK receptors alone, 5 min of exposure at 37 °C to gastrin (1 nM) resulted in the internalization of nearly 90% of type B CCK receptors.

Of interest, when co-expressed with type A CCK receptors in cells, type B CCK receptor internalization was significantly delayed with a prolonged time course. Using the same receptor agonist (1 nM Rho-nsCCK), no internalization was observed in 5 min, 50% internalization was finally observed after 10 min, and the internalization became almost complete by 15 min. Because receptor internalization represents a desensitization response of receptors, making them inaccessible to outside stimulus, the prolonged course of internalization would be reasonably assumed to result in longer response to stimulus and stronger cellular signal transduction. Internalization of FLAG-tagged type A CCK receptors was also studied using anti-FLAG antibody and FITC-conjugated goat anti-mouse second anti-

| CCK, Bmax | IC50 | CCK | G-17 | Rho-nsCCK | L-364,718 | L-365,260 |
|----------|------|-----|------|-----------|-----------|-----------|
| pmol/mg protein | nM  |     |      |           |           |           |
| CHO-CCKAR | 1.4 ± 0.2 | 5.4 ± 1.5 | >10^3 |           |           |           |
| CHO-CCKBR | 2.5 ± 0.8 | 4.1 ± 1.0 | 28 ± 6 | 57 ± 4    | 413 ± 12  | 15 ± 1    |
| CHO-CCKAR&CCKBR | 4.2 ± 1.0 | 7.9 ± 1.6 | 42 ± 7 | 23 ± 8    | 494 ± 140 | 85 ± 51  |

**Table I**

**Characterization of stable CHO cell lines expressing type A, type B, and both types of CCK receptors**

The binding parameters shown here represent the means ± S.E. of data from three to five independent experiments performed in duplicate.

**Table II**

**Characterization of tagged CCK receptor constructs used in BRET studies**

The data shown here represent the means ± S.E. from three to five independent experiments performed in duplicate.

| CCK binding Kd | Bmax | CCK activity EC50 |
|----------------|------|--------------------|
| pmol/mg        |      |                    |
| CCKAR          | 0.36 ± 0.1 | 0.5 ± 0.1 | 0.7 ± 0.2 |
| CCKAR-Rlu      | 0.10 ± 0.2 | 0.3 ± 0.1 | 1.9 ± 0.9 |
| CCKAR-YFP      | 0.53 ± 0.1 | 0.5 ± 0.1 | 1.8 ± 1.0 |
| CCKBR          | 0.81 ± 0.01 | 0.9 ± 0.05 | 0.3 ± 0.1 |
| CCKBR-Rlu      | 1.90 ± 0.9 | 0.4 ± 0.1 | 0.4 ± 0.1 |
| CCKBR-YFP      | 2.40 ± 0.5 | 2.1 ± 0.1 | 1.3 ± 0.1 |
body in permeabilized cells (Fig. 5). No internalization of type A CCK receptors was shown when expressed alone after incubation with Rho-naCCK (data not shown), consistent with the established very low affinity of this CCK analogue to bind to type A CCK receptors. When co-expressed with type B CCK receptors, in contrast, type A CCK receptors were shown to be internalized and to co-localize with type B CCK receptors in cytoplasmic vesicles after treatment with the same fluorescent CCK analogue. These data suggest that type B-type A CCK receptor heterodimers show novel receptor trafficking properties as compared with those of each receptor subtype alone.

**Agonist-induced Cell Proliferation**—Gastrin and CCK have been shown to be potent stimulators of cell growth (15, 16). To determine the role of heterodimerization on the trophic effect of these hormones, the cell proliferation responses to gastrin or CCK in cells expressing type B CCK receptors or type A CCK receptors or both were assayed and depicted in Fig. 6. As reported, gastrin modestly stimulated the growth of the cells expressing type B CCK receptors in a concentration-dependent manner, with a maximal response (1.3-fold over control) observed at 10 nM. Only the highest concentration of gastrin used stimulated growth in cells expressing type A CCK receptors (1.1-fold over control). Of interest, the gastrin-stimulated cell growth was significantly promoted in the type B-type A CCK receptor heterodimer-expressing cells, and 10 nM gastrin treatment resulted in increase of cell growth to 1.8-fold over control. Cells expressing either receptor alone or both receptors grew at a similar rate in serum-free medium without agonist treatment (data not shown). CCK had trophic effects analogous to those of gastrin in these cells (Fig. 6).

**DISCUSSION**

Receptor dimerization has recently been described to occur in G protein-coupled receptors (3–13), although what receptors are involved, how frequent this is, and what the functional significance might be are not yet clear. We previously used BRET to demonstrate that type A CCK receptors exist as constitutive homodimers when expressed in COS cells and that CCK binding leads to the dissociation of such complexes (1). Here, we used the same technique to explore whether type B CCK receptors also could exist as oligomers. Indeed, our results demonstrated that type B CCK receptors form homodimers, as well as heterodimers with type A CCK receptors when co-expressed on the same cell. The type A and type B CCK receptor heterodimers display new and unusual ligand binding, calcium response, and receptor trafficking properties. Moreover,
our data show that these heterodimer-expressing cells exhibit increased cell growth. Thus, our results provide the first evidence of interaction between type A and type B CCK receptors, along with unique and potentially quite important functional consequences.

The physiological and pharmacological activities of CCK and gastrin that have been observed are complex and have been difficult to understand based on the existence of only type A and type B CCK receptors. Examples of this include the observations that neither type A nor type B CCK receptor antagonists are able to antagonize the anti-nociceptive action of CCK in the mouse (28), and the spectrum of antagonist affinities for CCK receptors on the AR4–2J rat pancreatic tumor cell line is not consistent with binding to either type A or type B CCK receptors (14). To better explain these observations, the existence of additional subtypes of CCK receptors has been predicted. However, despite intensive efforts by several labs, only these two CCK receptor subtypes have been identified to date (29).

Of interest, heterodimerization between two structurally similar receptors has been described to result in pharmacological characteristics different from those of the individual receptors (8–10, 12). In the current study, we found that both type A and type B CCK receptor antagonists had low affinities for type A and type B CCK receptor heterodimers. Further, in heterodimer-expressing cells, agonist-induced calcium responses were poorly inhibited by both antagonists. Indeed, type A and type B CCK receptors have been found to co-localize in select

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**TABLE III**

| Ligand | Peptide concentration | CCKBR-Rlu/CCKBR-YFP (homodimer) | CCKAR-Rlu/CCKBR-YFP (heterodimer) |
|--------|-----------------------|---------------------------------|----------------------------------|
|        |                       | 0.11 ± 0.01                     | 0.10 ± 0.01                      |
|        | None                  | 0.10 ± 0.01                     | 0.10 ± 0.01                      |
| CCK-8  | 10⁻¹⁰                 | 0.10 ± 0.02                     | 0.10 ± 0.01                      |
|        | 10⁻⁸                  | 0.11 ± 0.02                     | 0.10 ± 0.01                      |
|        | 10⁻⁶                  | 0.10 ± 0.01                     | 0.10 ± 0.01                      |
| G-17   | 10⁻¹⁰                 | 0.10 ± 0.02                     | 0.10 ± 0.01                      |
|        | 10⁻⁸                  | 0.10 ± 0.02                     | 0.10 ± 0.01                      |
|        | 10⁻⁶                  | 0.10 ± 0.01                     | 0.10 ± 0.01                      |
areas of the brain where opioids have effects (30), and both of these receptors have been described to be expressed on AR4–2J cells (31). Our data suggest that type A and type B CCK heterodimers could provide an explanation for the above observations.

The molecular basis of the receptor dimerization may provide clues to better understand the changes in ligand specificity and action. It is believed that the peptide ligands for these receptors bind to extracellular loop and tail domains, whereas the benzodiazepine antagonists bind to intramembranous regions within the confluence of helical segments (32, 33). Two types of G protein-coupled receptor dimerization have been reported: domain-swapped dimers and contact dimers. In domain-swapped dimerization, a portion of one receptor molecule associates with the complementary domain of another receptor, whereas contact dimers form along the external surface of intact helical bundles. The current study demonstrates that the type A and type B CCK receptor heterodimers retain most of their peptide binding characteristics but exhibit a relative loss in their nonpeptidyl ligand binding. We postulate that the assembly of receptor domains in this heterodimer has formed a new antagonist binding pocket, while maintaining the peptidyl ligand-binding pocket relatively intact.

CCK and gastrin have been reported to stimulate cell proliferation in tissue culture cell models expressing type B CCK receptors (34) and a variety of cancer cell lines, including pancreatic, gastric, colonic, and small cell lung tumors (35, 36). A recent report of transgenic mice in which the type B CCK receptor is engineered to be expressed on acinar cells known to normally express only type A CCK receptors results in an unusual phenotype with proliferation and transdifferentiation of acinar cells (37). This further supports a link between cell growth with the simultaneous presence of both type A and type B CCK receptors.

However, prior to this, no evidence had been provided to support this hypothesis and address its molecular mechanism. The current study reveals that type A and type B CCK receptors can form constitutive heterodimers when co-expressed in living cells. Although the type A and type B CCK receptors may be present in both the brain and gastrointestinal tract (38) and even in the same healthy cell at distinct times during development (39), they are not normally present in a single healthy cell in the adult. One pathological state in which this has been postulated to occur is in tumors of the pancreas (40, 41). Considering the potential trophic effect of the natural agonists of these receptors in that setting, it is intriguing to postulate a pathophysiologic role for this heterodimerization.

Preeclampsia represents the first and, until now, the only human disorder reported to be associated with altered G protein-coupled receptor heterodimerization (42). In that syndrome, heterodimers including angiotensin-1 and bradykinin-2 receptors mediate enhanced angiotensin responsiveness (42). Here, we provide an analogous story for the role of G protein-coupled receptor heterodimerization that could have a profound effect on a more chronic function, such as cell growth.

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