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Pattern of Intra-Family Hetero-Oligomerization Involving the G-Protein-Coupled Secretin Receptor

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Abstract

Oligomerization of G-protein-coupled receptors (GPCRs) is emerging as a mechanism for regulation and functional modification, although it has been studied most extensively for Family A receptors. Family B receptors have clear structural differences from Family A. In this paper, we have systematically evaluated GPCRs that are capable of association with the prototypic Family B secretin receptor. All of the receptor constructs were shown to traffic normally to the plasma membrane. We utilized receptor bioluminescence resonance energy transfer (BRET) to determine the presence of constitutive and ligand-dependent receptor association. Extensive intra-family and no cross-family association was observed. Of the nine Family B receptors studied, all constitutively yielded a significant BRET signal with the secretin receptor, except for the calcitonin receptor. Each of the associating hetero-oligomeric receptor pairs generated a BRET signal of similar intensity, less than that of homo-oligomeric secretin receptors. BRET signals from some receptor pairs were reduced by ligand occupation, but none were increased by this treatment. Thus, Family B GPCR oligomerization occurs, with many structurally related members associating with each other. The specific functional implications of this need to be further evaluated.

Keywords

G-protein-coupled receptors; Heteroligomerization; Secretin receptor; Bioluminescence resonance energy transfer; Fluorescence resonance energy transfer; Surface expression

Introduction

Oligomerization of membrane receptors was first described for single transmembrane tyrosine kinase receptors in which such molecular association allows one receptor to

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phosphorylate the partner, a critical step in its function (Dawson et al. 2005). For the superfamily of heptahelical G-protein-coupled receptors (GPCRs), association in the plasma membrane has also been described (Terrillon and Bouvier 2004), although the frequency of this in normal physiology and in various disease states and its functional significance are far less clear (Park et al. 2004). GPCR oligomerization has been reported to have effects on the specificity of ligand binding, on ligand binding affinity, on signaling, and on trafficking (Cheng et al. 2003; Hague et al. 2006; Milligan 2004).

Structural similarity between associating receptors seems to represent an important determinant for their oligomerization. Indeed, many GPCRs are able to associate with themselves (homo-oligomerization). While hetero-oligomerization has been less extensively studied, most of the convincing reports of GPCR hetero-oligomerization involve structurally closely related receptors.

The current project was designed to explore possible rules for GPCR oligomerization by systematically studying the ability of a single receptor, the secretin receptor, to associate with a broad variety of other receptors. We have focused on Family B GPCRs, since we previously demonstrated that the secretin receptor does not associate with selected Family A GPCRs (Cheng and Miller 2001; Harikumar et al. 2006b). We have demonstrated the ability of this prototypic Family B GPCR to associate with itself (Harikumar et al. 2006b) and its ability to associate with the most closely related Family B GPCRs, receptors for vasoactive intestinal polypeptide (VIP), the VPAC1 and VPAC2 receptors (Harikumar et al. 2006b). We now extend the analysis of possible oligomerization of the secretin receptor to more distant Family B GPCRs. We have also studied the effect of ligand binding on the state of oligomerization of these receptors. This provides an extensive view of the potential for oligomeric association across a GPCR family.

Experimental Procedures

Materials

Dulbecco's modified Eagle's media (DMEM) and antibiotics were purchased from Invitrogen (Carlsbad, CA, USA), and Fetal Clone II medium supplement was from HyClone Laboratories (Logan, UT, USA). Growth hormone (1–40), GLP-1 [preproglucagon (7–36)], GLP-2 [preproglucagon (126–159)], parathyroid hormone(1–34), and α-calcitonin gene-related peptide(19–37) (CGRP) were purchased from Bachem (Torrance, CA, USA). Coelenterazine *h* was from Biotium (Hayward, CA, USA).

Receptor Constructs

Receptor carboxyl-terminal fusion constructs with *Renilla* luciferase (Rlu) and yellow fluorescent protein (YFP) were prepared using Gateway technology by Invitrogen (Carlsbad, CA, USA; Harikumar et al. 2006b). The destination vectors were generated using the Gateway Vector conversion system. The pCR3.1-Rlu destination vector was created by inserting the ccd (attR1/R2) cassette B 5′ to the Rlu complementary DNA (cDNA). Similarly, pEYFP destination vector was created by inserting the ccd (attR1/R2) cassette B into the EcoRV site of the vector 5′ to the YFP cDNA. The pEYFP construct was prepared by substituting codons for four amino acids (Ser^{66}-Gly, Val^{69}-Leu, Ser^{73}-Ala, and Thr^{204}-Tyr) into $pGFP^2$-N1 (Perkin Elmer, Wellesley, MA, USA). The cDNAs encoding each human receptor ($VPAC_1$, $VPAC_2$, PTH_1, PTH_2, GLP_1, GLP_2, CLR, CTR, and GHRH receptors) were amplified with Expand High Fidelity Enzyme blend (Roche) using a forward primer that introduced four bases (CACC) immediately before the ATG initiation codon and a reverse primer that removed the receptor's native stop codon. The PCR products were subcloned into the pENTR/D-TOPO vector using the pENTR directional

TOPO Cloning Kit (Invitrogen), following the manufacturer's recommendations. The recombination reactions between pENTR-specific receptor cDNA, and the destination vectors (pCR3.1-Rlu-dest and pEYFP-N1-dest) were performed using the LR recombinase kit. We previously described the construction of the human secretin receptor fusion constructs (Ding et al. 2002). All sequences were verified by direct DNA sequencing.

Cell Culture and Transient Receptor Expression

African monkey kidney (COS) cells were grown in DMEM supplemented with 5% *v*/*v* Fetal Clone II and were maintained at 37°C in a humidified atmosphere of 5% CO_2. COS cells were plated at a density of 0.5×10^6 cells per 10 cm tissue culture dish 24 h before transfection. Cells were transiently transfected with 3 μg of either donor construct alone or a combination of donor and acceptor constructs (total DNA concentration used was 3 μg/per dish) using a previously described diethylamino ethanol-dextran procedure (Harikumar et al. 2006a). The tagged receptor-bearing cells were used for microscopy or resonance energy transfer studies 48 h post-transfection.

Evaluation of Cell Surface Expression with Fluorescence Microscopy

COS cells expressing YFP-tagged receptor constructs were washed with phosphate-buffered saline (PBS) and fixed in 2% *w*/*v* formaldehyde in PBS for 30 min at room temperature. Cells were mounted on slides in Vectashield (Vector Laboratories, Burlingame, CA, USA). YFP fluorescence was observed, and images were collected using a Zeiss (Thornwood, NY) LSM 510 confocal microscope (excitation, 488 nm argon laser; emission, LP505 filter; pinhole diameter 2.2 airy units, Plan-Apochromat 63X/1.4NA oil). Background-subtracted images were prepared with Adobe Photoshop 7.0 (Mountain View, CA, USA).

BRET Studies

Fluorescently tagged receptor-bearing COS cells were detached from the tissue culture flasks using non-enzymatic cell-dissociation solution (Sigma). The cells were washed with Krebs–Ringers/4-2-hydroxyethyl-1-piperazineethanesulfonic acid (HEPES; KRH) medium (25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, and 1 mM KH_2PO_4) and were then suspended in KRH medium to yield a final concentration of approximately 10^6 cells/ml. BRET assays were performed as described previously (Harikumar et al. 2006b) with receptor-bearing cells in their unstimulated state or after stimulation with specific ligands at 37°C for 2 min. The cell-permeant *Renilla* luciferase substrate, coelenterazine *h*, was added to 1 million cells in a 1-ml quartz cuvette to yield a final concentration of 5 μM. Bioluminescence emission was immediately monitored in a SPEX FluoroMax-3 spectrofluorometer (SPEX Industries, Edison, NJ, USA) in the spectral range between 400 and 600 nm using wavelength increments of 2 nm and an integration time of 2 s. The fluorescence properties of YFP were measured by exposing cells expressing only the YFP-tagged receptors to light at 480 nm and scanning for emission in the spectral range from 500 and 580 nm. The BRET ratio was defined as [(emission at 510–580)−(emission at 440–500)×Cf]/(emission at 440–500), where Cf corresponds to (emission at 510–580)/(emission at 440–500) for the Rlu-tagged receptors expressed by themselves in analogous experiments.

FRET Studies

These studies were performed in manner analogous to the BRET studies, as we have previously reported (Lisenbee et al. 2007). Steady-state fluorescence intensities were acquired in a Fluoromax-3 fluorometer (SPEX industries, Edison, NJ, USA) at 25°C using a 1 ml quartz cuvette. Fluorescence resonance energy transfer between CFP-tagged receptor and YFP-tagged receptor was collected by exciting the samples at 433 nm and collecting the

emission spectra from 450 to 600 nm. Untransfected cells and medium were utilized to correct for background fluorescence.

Statistical Analyses

Comparison of independent samples was performed by analysis of variance, with *P* values<0.05 considered to be significant.

Results

Figure 1 illustrates the structural relationship between the Family B GPCRs that were studied. Shown is a proposed phylogenetic tree indicating the most plausible evolutionary relationship between these receptors. Also shown is the amino acid sequence alignment for the fourth transmembrane segment (TM IV) of these receptors, recently shown to be the most critical determinant of homo-oligomerization of the secretin receptor (Harikumar et al. 2007). COS cells transfected with the YFP-tagged receptor constructs were imaged using confocal fluorescence microscopy to examine their ability to traffic normally to the plasma membrane. Figure 2 shows representative images of cells documenting that YFP fluorescence was present at the level of the plasma membrane and within intracellular biosynthetic compartments for each of the receptor constructs. Quantitative analysis of surface YFP fluorescence using Metamorph (Molecular Devices, Sunnyvale, CA, USA) established that the levels of expression of each of the Family B receptors were not different from each other.

As previously observed, the secretin receptor formed homo-oligomers and hetero-oligomers with the VPAC1 and VPAC2 receptors as demonstrated by BRET (Fig. 3a). Analogous studies using the Family A cholecystokinin receptor demonstrated no significant BRET signal above that observed with a series of important negative controls (0.030); these included the co-expression of tagged secretin receptor with the complementary soluble tag or with the absence of the complementary donor or acceptor. Analogous experiments were also performed with a wide range of Family B GPCRs (Fig. 3a). It is noteworthy that all tested receptors in this family except for the calcitonin receptor yielded a positive BRET signal with the secretin receptor. Each of the pairs of receptors that provided a positive hetero-oligomeric BRET signal with the secretin receptor yielded a signal of similar intensity that was significantly lower ($p<0.05$) than that generated by the homo-oligomeric secretin receptor pair. This may suggest that the propensity to form homo-oligomers is greater than that to form hetero-oligomers.

Further evidence for absence of hetero-oligomerization between the secretin receptor and the calcitonin receptor was established through FRET measurements (Fig. 3b). This was performed with both combinations of CFP- and YFP-tagged secretin and calcitonin receptors. There was no significant FRET signal observed for hetero-oligomerization of this pair of receptors (FRET ratio of 0.10±0.01 for CFP-SecR and YFP-CTR and 0.12±0.01 for CFP-CTR and YFP-SecR, both below the background level of 0.12± 0.01), while a strong FRET signal was observed for homo-oligomerization of the secretin receptor used as a positive control (FRET ratio of 1.08±0.2).

The effect of ligand binding on GPCR oligomerization has varied from augmenting the oligomerization process to disrupting the oligomers, and to having no effect (Cheng et al. 2003; Cheng and Miller 2001; Ding et al. 2002; Harikumar et al. 2006b; Rocheville et al. 2000b). We previously observed no effect of secretin on constitutive homo-oligomerization of the secretin receptor (Harikumar et al. 2006b). In contrast, VIP was observed to reduce resonance energy transfer from constitutive homo-oligomers of the VPAC1 or VPAC2 receptors or hetero-oligomers made up of VPAC1 and VPAC2 receptors (Harikumar et al.

2006b). In contrast, neither VIP nor secretin-modified resonance energy transfer from hetero-oligomers made up of the secretin and VPAC receptors (Harikumar et al. 2006b).

Figure 4 illustrates the BRET ratios after ligand binding to each of the secretin receptor hetero-oligomeric complexes. Treatment of cells bearing GLP_1/secretin or GLP_2/secretin receptor hetero-oligomers with GLP-1, GLP-2, or secretin had no effect on the BRET signal. BRET signals from GHRH/secretin and CLR/secretin receptor hetero-oligomers were unaltered by the treatment of any of the ligands studied in this paper. In contrast, parathyroid hormone treatment significantly reduced the BRET signal for each of the PTH/secretin hetero-oligomeric receptor complexes. Secretin reduced the BRET signal only for the PTH_2/secretin hetero-oligomeric receptor complex.

Discussion

GPCR oligomerization is now well recognized, with the potential for significant functional and regulatory impact (Cvejic and Devi 1997; Pascal and Milligan 2005; Rocheville et al. 2000a). The most extensive analysis of GPCR oligomerization has been performed on Family A receptors, and this receptor subfamily is also the best characterized with regard to structure (Milligan 2007). Family B GPCRs also form homo-oligomeric (Harikumar et al. 2006b; Lisenbee and Miller 2006; Seck et al. 2003) and hetero-oligomeric complexes (Harikumar et al. 2006b). The structure of receptors in this family has been predicted to be distinct from those in Family A (Dong et al. 2007; Salom et al. 2006), with different signature sequences and even predicted differences in the helical bundle.

Family B GPCRs have a long amino-terminal extracellular tail region that includes six conserved cysteine residues that are involved in three conserved disulfide bonds (Lisenbee et al. 2005). This region is important for natural peptide ligand binding (Dong and Miller 2002). This region, however, is not important for secretin receptor oligomerization (Lisenbee and Miller 2006). The intracellular carboxyl-terminal tail of the secretin receptor can also be truncated without effect on its oligomerization (Lisenbee and Miller 2006). In a recent study, the major determinant for secretin receptor homo-oligomerization was the lipid-exposed face of the fourth transmembrane segment (Harikumar et al. 2007).

In the current work, we have systematically explored the ability of a broad variety of Family B GPCRs to form hetero-oligomeric complexes with the secretin receptor. We already knew that selected Family A GPCRs did not form such complexes (Harikumar et al. 2006a) and that the most closely related Family B GPCRs, the VPAC1 and VPAC2 receptors, were able to form oligomers (Harikumar et al. 2006b). In this studies, we extended this survey across a wide range of Family B peptide hormone receptors. Of note, each of these receptors except for the calcitonin receptor was able to form hetero-oligomers with the secretin receptor indicating that the potential for hetero-oligomerization is likely to be extensive across Family B receptors. In evaluating the fourth transmembrane segment of these receptors, it is interesting that the human calcitonin receptor includes two residues within this segment that are not present in any other receptors in this series. These are Arg at equivalent position to Tyr^{233} of the secretin receptor and Thr at equivalent position to Ala^{250} of the secretin receptor; these residues are predicted to be at either end of the transmembrane segment, facing the lipid bilayer. One (or both) of these residues could contribute to the distinct hetero-oligomerization behavior exhibited by the calcitonin receptor in the current series of experiments. However, it is not clear if the same sequence determinant responsible for homo-oligomerization is also the primary sequence involved in hetero-oligomerization. Future work will address the physiological significance of heterologous interaction across Family B receptors.

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Abbreviations

BRET	bioluminescence resonance energy transfer
CLR	calcitonin receptor-like receptor
CTR	calcitonin receptor
FRET	fluorescence resonance energy transfer
GHRHR	growth hormone releasing hormone receptor
GLP_1R	type 1 glucagon-like peptide receptor
GLP_2R	type 2 glucagon-like peptide receptor
PTH_1R	type 1 parathyroid hormone receptor
PTH_2R	type 2 parathyroid hormone receptor
Rlu	*Renilla* luciferase
SecR	secretin receptor
VIP	vasoactive intestinal polypeptide
$VPAC_1R$	type 1 vasoactive intestinal polypeptide receptor
$VPAC_2R$	type 2 vasoactive intestinal polypeptide receptor
YFP	yellow fluorescent protein

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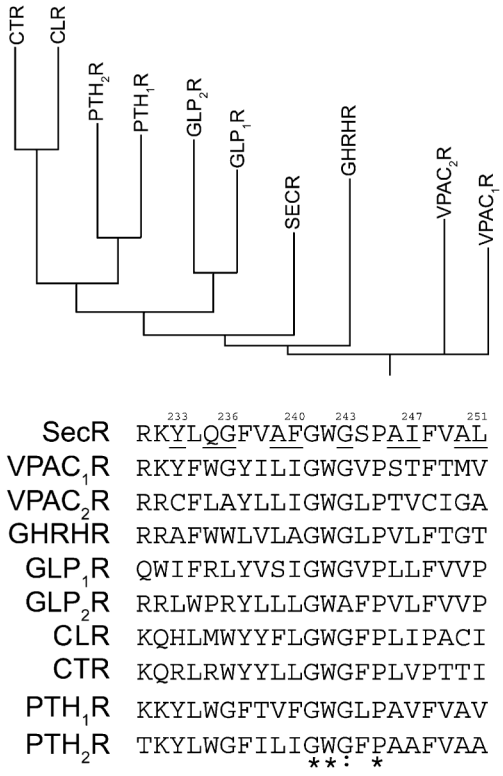

Figure 1.

Sequence analysis of the Family B GPCRs studied. A phylogenetic tree of Family B GPCRs was constructed using ClustalW in the Molecular Biology Work Bench web site (http://workbench.sdsc.edu) (*top panel*). The alignment of amino acid residues in the proposed fourth transmembrane segment of these receptors is also illustrated (*bottom panel*). Fully conserved residues are identified with an *asterisk*, with highly conserved residues identified with a *colon*. The numbering scheme utilized refers to residue number for the secretin receptor, with residues predicted to be lipid-exposed in the secretin receptor highlighted with *underlining*

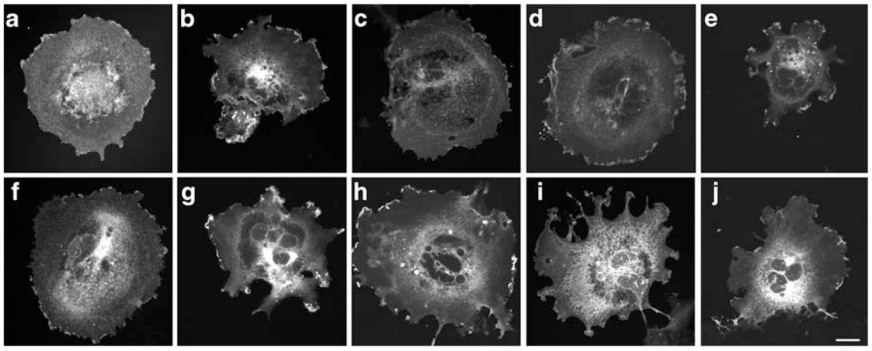

Figure 2.

Fluorescence localization of YFP-tagged Family B GPCRs. Shown are representative confocal fluorescence images of transfected COS cells. All the Family B receptor constructs were observed to efficiently sort to the plasma membrane, as demonstrated by YFP fluorescence in that cellular compartment, as well as in intracellular biosynthetic compartments. Quantitative analysis of YFP fluorescence at the cell surface using Metamorph demonstrated that there was no difference in plasma membrane expression of any of the receptor constructs. **a** SecR-YFP, **b** $VPAC_1R$-YFP, **c** $VPAC_2R$-YFP, **d** PTH_1R-YFP, **e** PTH_2R-YFP, **f** GLP_1R-YFP, **g** GLP_2R-YFP, **h** CTR-YFP, **i** CLR-YFP, **j** GHRHR-YFP. *Bar* 25 μm

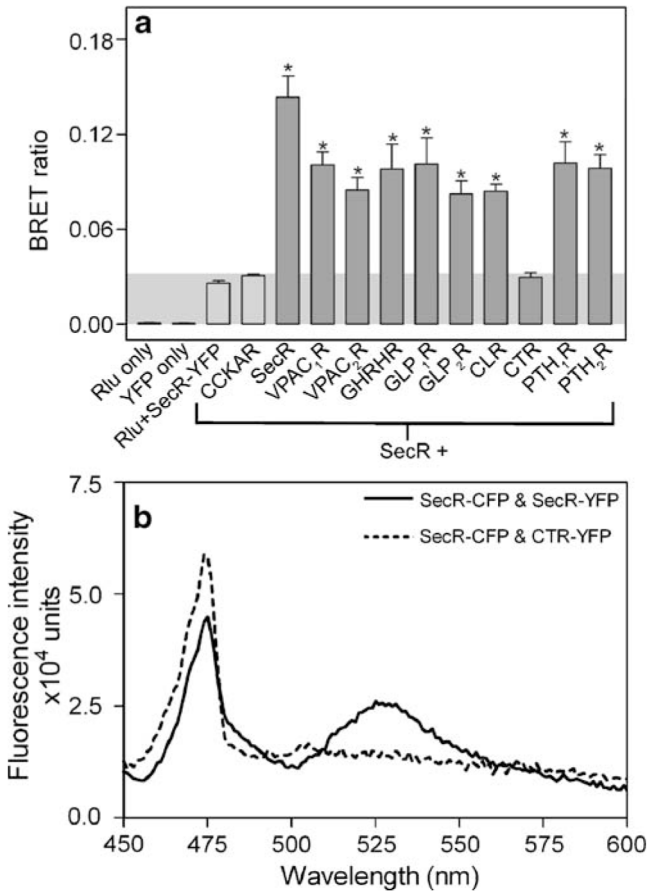

Figure 3.

Secretin receptor BRET. Shown in **a** are the BRET signals generated between the secretin receptor and other GPCRs. When two receptors are identified in a single lane, this reflects co-expression of complementarily labeled receptor constructs, with one tagged with Rlu and the other tagged with YFP. The *shaded area* represents the non-specific BRET signal obtained from the YFP-tagged human secretin receptor coexpressed with the structurally unrelated Rlu-tagged Family A cholecystokinin receptor or with soluble Rlu. All of the Family B GPCRs studied generated significant BRET signals above background, except for the calcitonin receptor. *Asterisks* represent resonance energy transfer signals significantly different from background at a level of $p<0.05$. Values represent means±SEM of data from four to six independent experiments. Shown in **b** are the patterns of fluorescence emission in the FRET studies with the secretin receptor and the calcitonin receptor. SecR-CFP and SecR-YFP provided a positive control, while SecR-CFP and CTR-YFP yielded no FRET signal above the background level. These studies provided further confirmation of the absence of significant BRET between these receptors

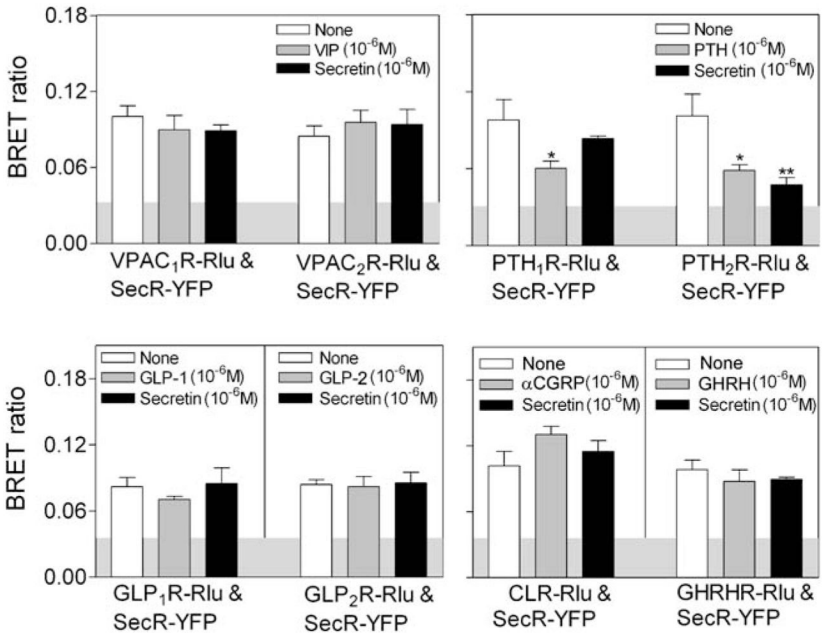

Figure 4.

Effect of ligand binding on secretin receptor BRET. Shown are the BRET signals generated in the absence and presence of receptor ligands. There was no significant change in the BRET signal after ligand occupation for hetero-oligomeric complexes involving the secretin receptor, except for that including the PTH receptors where the signal was reduced. *Asterisk* represent BRET signals in the presence of ligand that were significantly different from the control unstimulated signals at the level of $p<0.05$. Values represent means± SEM of data from four to six independent experiments