

# Constitutive Formation of Oligomeric Complexes between Family B G Protein-Coupled Vasoactive Intestinal Polypeptide and Secretin Receptors

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## ABSTRACT

Formation of oligomeric complexes of family A G protein-coupled receptors has been shown to influence their function and regulation. However, little is known about the existence of such complexes for family B receptors in this superfamily. We previously used bioluminescence resonance energy transfer (BRET) to demonstrate that the prototypic family B secretin receptor forms ligand-independent oligomeric complexes. Here, we show that subtypes of human vasoactive intestinal polypeptide receptors (VPAC1 and VPAC2) that represent the closest structurally related receptors to the secretin receptor also form constitutive oligomers with themselves and with the secretin receptor. We prepared tagged constructs expressing *Renilla reniformis* luciferase, yellow fluorescent protein, or cyan fluorescent protein at the carboxyl terminus of VPAC1, VPAC2, and secretin receptors, and performed BRET and morphologic fluorescence resonance energy transfer (FRET) studies with all combinations. The specificity of the BRET and FRET signals was confirmed by control studies. These constructs bound their natural ligands specifically and saturably, with these ago-

nists able to elicit full cAMP responses. BRET studies showed that, like the secretin receptor, both VPAC receptors exhibited constitutive homo-oligomerization in COS cells. Unlike secretin receptor oligomers that were unaffected by ligand binding, the VPAC receptor homo-oligomers were modulated by vasoactive intestinal polypeptide. In addition, each of these three receptors formed hetero-oligomers with each other. The VPAC1-VPAC2 hetero-oligomers were modulated by vasoactive intestinal polypeptide binding, whereas the secretin-VPAC1 and secretin-VPAC2 receptor hetero-oligomers were unaffected by ligand treatment. Morphologic FRET studies demonstrated that each of the homo-oligomers and the VPAC1-VPAC2 receptor hetero-oligomers reached the cell surface, where receptor interactions were clear. However, coexpression of secretin receptors with either type of VPAC receptor resulted in intracellular trapping of the hetero-oligomeric complexes within the biosynthetic pathway. These studies provide new insight into the ability of family B G protein-coupled receptors to associate with each other in cells.

Plasma membrane receptors represent a critically important interface between circulating and extracellular hormones and neurotransmitters and the cell's intracellular sig-

naling cascades and effector machinery. We are beginning to recognize that the specificity and activity of ligand-receptor interaction at this critical interface can be affected by a variety of factors, including covalent modifications of the receptor and bimolecular interactions with it. One group of such interactions is the dimerization or oligomerization with the same or other receptors (Bouvier, 2001; Milligan, 2004; Park et al., 2004). This theme has been thoroughly explored for single transmembrane receptors (Overton et al., 2003), but it is also relevant to the heptahelical G protein-coupled receptors both in physiologic and pathologic settings (Ding et

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**ABBREVIATIONS:** BRET, bioluminescence resonance energy transfer; FRET, fluorescence resonance energy transfer; ALPHA, amplified luminescent proximity homogenous assay; DMEM, Dulbecco's modified Eagle's medium; VIP, vasoactive intestinal peptide; Rlu, *R. reniformis* luciferase; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; SecR, secretin receptor; PCR, polymerase chain reaction; VPAC1R, type 1 vasoactive intestinal polypeptide receptor; VPAC2R, type 2 vasoactive intestinal polypeptide receptor; EYFP, enhanced yellow fluorescent protein; BSA, bovine serum albumin; PBS, phosphate-buffered saline; CCK, cholecystokinin; RAMP, receptor activity-modifying family of protein; PG97-269, (Ac-His<sup>1</sup>, D-Phe<sup>2</sup>, Lys<sup>15</sup>, Arg<sup>16</sup>, Leu<sup>27</sup>)VIP.

al., 2002; Fotiadis et al., 2003). This can involve homo-oligomerization of like receptors or hetero-oligomerization of distinct receptors; these latter interactions can even result in agonist selectivity different from that of either of the components (Cheng and Miller, 2001; Cheng et al., 2003). These processes have been most extensively examined for family A G protein-coupled receptors (Angers et al., 2000; Rocheville et al., 2000; Ayoub et al., 2002; Carrillo et al., 2003; Cheng et al., 2003). For these, a broad variety of themes have been described, including oligomerization that can be constitutive or ligand-dependent, and ligand interaction that can have no effect or even dissociate such complexes (Kroeger et al., 2001; McVey et al., 2001; Ding et al., 2002; Mercier et al., 2002; Gomes et al., 2002; Latif et al., 2002; Cheng et al., 2003; Terrillon et al., 2003; Pflieger and Eidne, 2005). Potential effects that have been described include effects on the selectivity of ligand binding, G protein recognition, control of the signal transduction cascade, and receptor desensitization (George et al., 2000; Carrillo et al., 2003).

Although family B G protein-coupled receptors share their heptahelical architecture and G protein-coupling with family A G protein-coupled receptors, they are structurally distinct, lacking the signature sequences typical of the family A receptors and having their own unique features (Dong and Miller, 2002). Among the most characteristic features of family B receptors is a relatively long amino-terminal tail that contains six highly conserved cysteine residues known to contribute to three highly conserved disulfide bonds (Qi et al., 1997; Lisenbee et al., 2005). These structural constraints undoubtedly contribute to a stable structure that is critical for natural peptide ligand binding and action (Dong et al., 2004).

Much less is currently understood about the oligomerization of family B G protein-coupled receptors. It is now clear that the prototypic secretin receptor, the first family B G protein-coupled receptor to be cloned (Ishihara et al., 1991), is present as a constitutive homo-oligomer in the plasma membrane of living cells (Ding et al., 2002). In the current work, we examine the two receptors structurally most closely related to the secretin receptor, the receptors that bind vasoactive intestinal polypeptide with high affinity (VPAC1 and VPAC2 receptors) (Laburthe et al., 2002). For each of these receptors, we explore their ability to oligomerize in living cells using bioluminescence resonance energy transfer (BRET), both with themselves and with their related partners. In addition, we examine the effect of binding each of the natural ligands of these receptors and an antagonist on these processes. In an effort to better understand the differential ligand dependence of the oligomers, we also performed morphologic fluorescence resonance energy transfer (FRET) to explore cellular compartments in which receptor association was occurring. Although each receptor was capable of trafficking normally to the cell surface, coexpression of the secretin receptor with either of the VPAC receptors resulted in intracellular trapping of hetero-oligomeric complexes.

## Materials and Methods

**Materials.** Vasoactive intestinal polypeptide was purchased from Auspep (Parkville, VIC, Australia). Bovine serum albumin and isobutyl methylxanthine were from Sigma-Aldrich (St. Louis, MO),

and amplified luminescent proximity homogenous assay (ALPHA)-screen cAMP kits were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Dulbecco's modified Eagle's medium (DMEM) and HEPES were from Invitrogen (Carlsbad, CA); fetal bovine serum was from Trace (BioSciences, Sydney, Australia), and fetal clone II was from Hyclone Laboratories (Logan, UT). Formaldehyde was purchased from Ted Pella (Redding, CA). Cell culture plasticware was from NUNC A/S (Roskilde, Denmark), and Metafectine was from Scientifix (Cheltenham, VIC, Australia).  $^{125}\text{I}$ -VIP was prepared with Na- $^{125}\text{I}$  (100 mCi/ml) (MP Biomedicals, Irvine, CA) using IODO-BEADS (Pierce Chemical, Rockford, IL), with the monoiodinated peptide purified by reversed-phase high performance liquid chromatography.

**cDNA Constructs.** The *Renilla reniformis* luciferase (Rlu), yellow fluorescent protein (YFP), and cyan fluorescent protein (CFP) fusion constructs of VPAC1 and VPAC2 receptors were generated using Gateway technology by Invitrogen (Carlsbad, CA). The destination vectors were generated in-house using the Gateway Vector conversion system. In brief, the Rlu gene was PCR-amplified, and TA was cloned into the pCR3.1 vector. Immediately 5' to the Rlu gene, the ccd (attR1/R2) cassette B (Invitrogen) was blunt-end cloned. A pEYFP construct was created in-house by substituting four amino acids in the pGFP<sup>2</sup>-N1 vector (PerkinElmer Life and Analytical Sciences, Boston, MA): Ser<sup>66</sup>-Gly, Val<sup>69</sup>-Leu, Ser<sup>73</sup>-Ala, and Thr<sup>204</sup>-Tyr. The pEYFP destination vector was created by inserting the ccd (attR1/R2) cassette B into the EcoRV site of the vector, 5' to the YFP gene. A CFP destination vector was prepared in a similar manner by substituting in pGFP<sup>2</sup>-N1 amino acids Phe<sup>65</sup>-Leu, Ser<sup>66</sup>-Thr, Tyr<sup>67</sup>-Trp, Asn<sup>147</sup>-Ile, Met<sup>154</sup>-Thr, and Val<sup>164</sup>-Ala. cDNA encoding the VPAC1 and VPAC2 receptors were amplified with Expand High Fidelity Enzyme blend (Roche Diagnostics, Indianapolis, IN) 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 blunt-end PCR products were cloned into the pENTR/D-TOPO vector using the pENTR Directional TOPO cloning kits (Invitrogen) according to manufacturer's recommendations. Subsequent LR recombination reactions between pENTR-VPAC1 or pENTR-VPAC2 and the destination vectors pCR3.1-Rlu-dest, pEYFP-N1-dest, or pECFP-N1-dest were performed using the LR recombination kit following the manufacturer's recommendations. Construction of human secretin receptor constructs having Rlu or YFP fused at the carboxyl-terminal end was described previously (Cheng and Miller, 2001). A CFP-tagged SecR was created by TA cloning into pCRII (Invitrogen), a PCR-amplified CFP fragment from pECFP-N1 (BD Biosciences Clontech, Palo Alto, CA) that incorporated in-frame XhoI and XbaI sites immediately 5' of the start codon and 3' of the stop codon, respectively, to yield pCRII-XhoI-ECFP-XbaI. The XhoI/XbaI fragment from this construct was ligated into XhoI/XbaI-digested pcDNA3-SecR-YFP to effectively swap fluorescent protein coding sequences and yield pcDNA3-SecR-CFP. All sequences were verified by direct DNA sequencing.

**Cell Culture and Transfection.** COS cells were maintained in DMEM supplemented with either 5% (v/v) fetal bovine serum or 5% (v/v) fetal clone II and maintained at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. For cAMP and binding assays, cells were seeded one day before transfection into either 48 (1-cm<sup>2</sup>)-well plates or six (8-cm<sup>2</sup>)-well plates at  $\sim 125 \times 10^3$  cells/well and  $10^6$  cells/well, respectively. The following day, when cells were 90 to 100% confluent, they were transfected using 0.75  $\mu\text{l}/\text{cm}^2$  Metafectine and 50 to 100 ng/cm<sup>2</sup> DNA, as per the manufacturer's instructions. Lipid complexes were allowed to form for 30 min before being added to cells containing serum-free DMEM. After 5-h incubation, an equal volume of DMEM containing 5% fetal bovine serum was added. Cells to be used in radioligand binding were incubated under growth conditions for a further 36 h, whereas cells used for cAMP measurement were incubated overnight before being serum-starved for an additional 24 h. For BRET assays

and fluorescence microscopy, cells were plated at a density of  $5 \times 10^5$  cells/dish in 10-cm Petri dishes 1 day before transfection. The transfection procedure was performed by the previously established DEAE-dextran method using a total DNA concentration of 3  $\mu$ g per 10-cm dish (Cheng and Miller, 2001).

**Measurement of cAMP.** Cells were harvested, counted, and diluted to  $10^4$  cells/5  $\mu$ l in phenol red-free DMEM containing 0.1% (w/v) bovine serum albumin (BSA) and 1 mM isobutyl methylxanthine (stimulation buffer), and incubated for at least 30 min at 37°C. Agonist dilutions were prepared in stimulation buffer with 5  $\mu$ l added to white Opti-384-well plates (PerkinElmer Life and Analytical Sciences), with each point repeated in triplicate. After the 30-min cell incubation,  $10^4$  cells were added per well in a volume of 5  $\mu$ l. The plates were briefly centrifuged and then incubated for 30 min at 37°C. Agonist-stimulated receptor activity was terminated by the addition of 10  $\mu$ l of lysis buffer [5 mM HEPES, pH 7.4, 0.3% (v/v) Tween 20, and 0.1% (w/v) BSA]. The cAMP level was assayed in the same wells using ALPHA-screen assay kits. In brief, cAMP was measured with light-sensitive acceptor and donor beads that were prepared in lysis buffer and added to plates according to manufacturer's instructions. After overnight incubation in the dark, the plates were read with an ALPHA-screen protocol on a Fusion plate reader (PerkinElmer Life and Analytical Sciences). Data were analyzed using Prism 4 software (GraphPad Software Inc., San Diego, CA). In each assay, the quantity of cAMP generated was calculated from the raw data using a cAMP standard curve.

**Radioligand Binding.** Cells transfected in 48-well plates and incubated for approximately 36 h were assayed for  $^{125}$ I-VIP receptor binding. Cells were incubated in binding buffer [DMEM with 0.3% (w/v) BSA] containing approximately 120 pM  $^{125}$ I-VIP in the absence (total binding) or presence of increasing concentrations of unlabeled peptide. Cells were incubated for 1 h at 37°C before being washed with 250  $\mu$ l of PBS and then solubilized with 250  $\mu$ l of 0.5 M NaOH. The cell lysate was collected and counted in a PerkinElmer  $\gamma$ -counter (75% efficiency) to determine bound radioactivity. Competition-binding data were analyzed via nonlinear regression using GraphPad Prism 4.

**BRET Studies.** Transiently transfected COS cells were detached from culture flasks 48 h after transfection with a nonenzymatic cell dissociation solution (Sigma-Aldrich). The cells were washed with Krebs-Ringers-HEPES solution [25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 1.2 mM  $\text{MgSO}_4$ , 2 mM  $\text{CaCl}_2$ , 1 mM  $\text{KH}_2\text{PO}_4$  plus 0.2% (w/v) BSA and 0.01% (w/v) soybean trypsin inhibitor] and then resuspended in Krebs-Ringers-HEPES to a final concentration of approximately  $10^6$  cells/ml. Where appropriate, aliquots of these cells were treated with various ligands at 37°C for 2 min before BRET assays that were performed as we described previously (Cheng and Miller, 2001). In brief, the cell-permeant *R. reniformis* luciferase substrate coelenterazine h (Biotium, Hayward, CA) was added to 1 million cells in a 1-ml quartz cuvette to a final concentration of 5  $\mu$ M. Bioluminescence emissions were monitored immediately in a SPEX FluoroMax-3 spectrofluorometer (SPEX Industries Inc., Edison, NJ) 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 exciting at 480 nm cells expressing YFP-tagged receptors alone and scanning for emission in the spectral range from 500 to 580 nm. Consistent with our previous work (Cheng and Miller, 2001), the BRET ratio was defined as [(emission at 510–580) – (emission at 440–500)  $\times$  Cf]/(emission at 440–500), where Cf corresponds to (emission at 510–580)/(emission at 440–500) for the Rlu-tagged VPAC or secretin receptors expressed alone in analogous experiments.

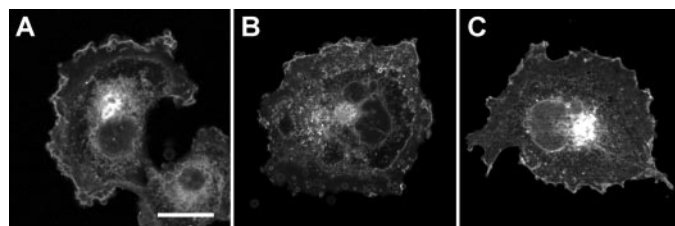
**Confocal and Morphologic FRET Microscopy.** Transfected cells were lifted from Petri dishes approximately 24 h after transfection and seeded to UV-sterilized 25-mm round coverslips in six-well plates. After culture for an additional 24 to 48 h, cells that adhered to the coverslips were washed once in PBS and then fixed in 2% (w/v) formaldehyde in PBS for 30 min at room temperature.

After two washes in PBS, the cells were mounted in VECTA-SHIELD (Vector Laboratories, Burlingame, CA) and then observed and photographed with a Zeiss LSM 510 confocal microscope (Carl Zeiss, Thornwood, NY) configured for YFP fluorescence (excitation, 514-nm argon laser; emission, LP530 filter; pinhole diameter, 1 airy unit; and objective, Plan-Apochromat 63 $\times$ /1.4 numerical aperture oil). Morphologic FRET imaging used an Axiovert 200M microscope (Carl Zeiss) equipped with dedicated CFP (excitation, 436/20 nm; dichroic, 455DCLP; and emission, 480/40 nm), YFP (excitation, 500/20 nm; dichroic, Q515LP; and emission, 535/30 nm), and FRET (excitation, 436/20 nm; dichroic, 455DCLP; and emission, 535/30 nm) epifluorescence filter sets (Chroma Technology Corp., Brattleboro, VT). Digital micrographs were collected separately from all three channels with a monochromatic ORCA-ER charge-coupled device camera (Hamamatsu, Bridgewater, NJ). Image acquisition was automated with QED InVivo software version 2.0 (Media Cybernetics, Inc., Silver Spring, MD). Exposure times ranging from 10 to 250 ms (no binning) were equivalent for all three channels collected from a given sample and were chosen objectively to both maximize signal depth and minimize pixel overexposure. FRET images were corrected for donor and acceptor bleed-through with MetaMorph version 6.3 (Molecular Devices, Sunnyvale, CA) using the sensitized emission method. Here, corrected FRET is defined as  $\text{FRETc} = \text{FRET} - (B \times \text{CFP}) - (A \times \text{YFP})$ , where FRET, CFP, and YFP represent background-subtracted images collected from the corresponding FRET, CFP, and YFP channels, and the coefficients B and A represent the proportion of the FRET signal attributable to CFP (donor) and YFP (acceptor) bleed-through. The latter were calculated from average threshold pixel intensities of cells expressing soluble CFP or YFP proteins alone (B, FRET/CFP; A, FRET/YFP) and were confirmed for cells expressing similarly tagged receptors. The bleed-through coefficients used were  $B = 0.55 \pm 0.01$  and  $A = 0.15 \pm 0.01$  and represent the means  $\pm$  S.E.M. for five separate data sets. Micrographs were assembled into figures and adjusted for contrast using Adobe Photoshop version 7.0 (Adobe Systems, Mountain View, CA), but it is noted that contrast adjustments to FRET images were minimal and were performed so as not to compromise the quantitative nature of these data.

**Statistical Analyses.** Data were analyzed using Student's *t* test for comparison of independent samples.

## Results

Each of the VPAC1, VPAC2, and secretin receptor constructs was tagged with one of three distinct fluorescent or luminescent proteins at its carboxyl terminus and then expressed in COS cells alone or in combination for functional and resonance energy transfer analyses. Figure 1 shows that each of the YFP-tagged receptors sorted prop-



**Fig. 1.** Morphologic demonstration of normal trafficking of fluorescently tagged receptors. Shown are confocal fluorescence micrographs (single optical sections) of formaldehyde-fixed COS cells expressing YFP-tagged versions of VPAC1 (A), VPAC2 (B), or secretin (C) receptors. Each of the three receptor constructs was localized throughout the biosynthetic cascade (endoplasmic reticulum and Golgi) and within the plasma membrane. Scale bar in A, 25  $\mu$ m.



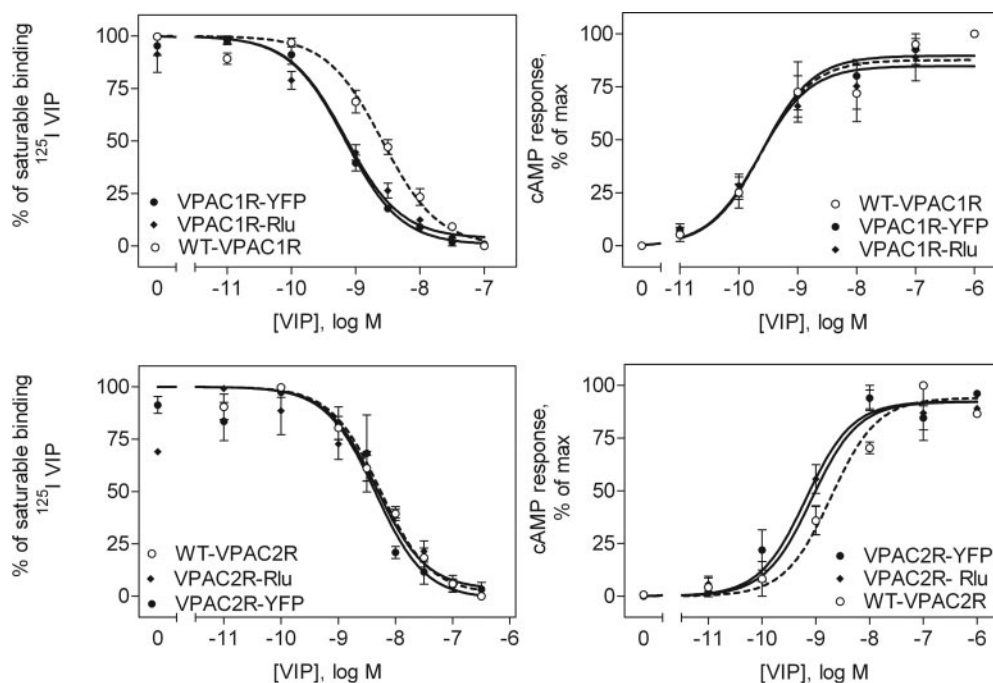
erly to the plasma membrane. A significant amount of intracellular fluorescence also was observed in the endoplasmic reticulum and Golgi cluster, probably because of the high levels of constitutive expression from the cytomegalovirus promoter.

The functional characteristics of these constructs were evaluated by performing competition-binding assays and determining biologic activity by measuring agonist-stimulated cAMP responses. The receptor-binding studies showed that both Rlu- and YFP-tagged VPAC1 and VPAC2 receptors bound vasoactive intestinal polypeptide specifically, saturably, and with high affinity, similar to wild-type VPAC receptors (Fig. 2; Table 1). VPAC receptors couple to  $G_s$  and are associated with adenylate cyclase signaling. All of the tagged VPAC receptor constructs showed concentration-dependent, vasoactive intestinal polypeptide-stimulated cAMP responses in receptor-expressing COS cells that were similar to those observed in wild-type receptors (Fig. 2; Table 1). This supports the observation that all of the tagged receptor constructs were fully functionally active in COS cells. We have reported the functional characterization of Rlu- and YFP-tagged secretin receptor constructs used in this study (Cheng and Miller, 2001). Both of these constructs were able to bind secretin peptide specifically, saturably, and with high affinity, typical of the wild-type construct, and were able to stimulate full cAMP responses using secretin.

Figure 3 shows the emission spectra of tagged VPAC receptor constructs when expressed in COS cells. The emission spectra of Rlu-tagged receptors illustrated a characteristic emission peak in the 475- to 480-nm range, denoting the

intensity of bioluminescence after exposure of the cells to coelenterazine h. The emission spectra of YFP-tagged receptor constructs illustrated a characteristic emission peak at 525 nm after excitation at 480 nm. Based on theoretical considerations, resonance energy transfer can occur when both donor and acceptor are present at a distance of 10 to 100 Å with dynamic orientation of the dipole. Indeed, when these constructs were coexpressed, resonance energy transfer occurred upon exposure of cells to coelenterazine h (Fig. 3). The donor Rlu protein showed a typical emission pattern that was able to excite the acceptor YFP protein, leading to emission of light of the expected wavelength. In the past, we used a soluble Rlu-YFP fusion protein construct as a positive control to demonstrate a significant BRET signal (BRET ratio of ~0.40) in cells expressing this construct (Cheng and Miller, 2001).

Because the BRET signal is small for receptor-bearing cells relative to the Rlu-YFP fusion protein (data not shown), it was important to rule out random, nonspecific interactions that may occur between coexpressed Rlu- and YFP-tagged membrane proteins. To verify and quantify such BRET signals, and to confirm that these signals were because of close spatial interactions, we performed a series of control studies. These included expressing Rlu- or YFP-tagged VPAC receptor constructs individually, or in combination with the alternately tagged cytosolic protein or structurally unrelated G protein-coupled receptor. The results of these control studies are shown in Fig. 4. The data show that cells coexpressing Rlu- and YFP-tagged VPAC receptors were able to form homo-oligomers and showed a clear BRET signal, with BRET



**Fig. 2.** Vasoactive intestinal polypeptide binding and biologic activity at receptor constructs. Shown are the vasoactive intestinal polypeptide competition-binding curves (left) and vasoactive intestinal polypeptide-stimulated cAMP responses (right) in COS cells expressing wild-type (WT) VPAC1 receptor or VPAC1 receptor constructs that include carboxyl-terminal fusions in-frame with Rlu or YFP (top), or cells expressing wild-type (WT) VPAC2 receptor or VPAC2 receptor constructs that include carboxyl-terminal fusions in-frame with Rlu or YFP (bottom). Binding data for each construct were normalized relative to control where no competing unlabeled vasoactive intestinal polypeptide was present, subtracting nonspecific binding from both  $[(B - N/B_0 - N) \times 100]$ . Specific binding at the VPAC1 receptor represented 85 to 90% for all constructs. Specific binding for the VPAC2 receptor was approximately 75% for WT and Rlu-fused receptors and 45% for YFP-fused receptor. The cAMP responses were normalized relative to the maximal response to vasoactive intestinal polypeptide in cells expressing each construct. Data represent the means  $\pm$  S.E.M. of three to four independent assays performed in duplicate.  $B$ ,  $^{125}$ I-VIP bound;  $B_0$ , binding in the absence of competing ligand; and  $N$ , nonspecific binding.

ratio values of approximately 0.11. There was little or no BRET signal (BRET ratio of  $\sim 0.02$ ) observed when cells expressed only one of the tagged receptors, or a tagged receptor and the opposite BRET partner as a soluble protein. This was also true when structurally distinct and fully functional YFP-tagged human cholecystokinin (CCK) receptor was coexpressed with Rlu-tagged VPAC1 or VPAC2 receptors. Thus, BRET ratio values of  $\sim 0.02$  to 0.03 were empirically defined as background, and only values above 0.06 were considered to be significant for this study.

Figure 5 shows the results of BRET experiments aimed at determining whether structurally related VPAC1 or VPAC2 receptors can form oligomers with themselves and with secretin receptors. Significant BRET signals above background were observed when Rlu-tagged versions of VPAC1, VPAC2, or secretin receptors were expressed with YFP-tagged versions of the same receptor (Fig. 5A). These data support the abilities of VPAC1, VPAC2, and secretin receptors to constitutively form homo-oligomers in living cells. In addition, significant BRET signals were also observed when VPAC1R-Rlu/VPAC2R-YFP, VPAC1R-Rlu/SecR-YFP, or VPAC2R-Rlu/SecR-YFP were coexpressed (Fig. 5B), indicating that constitutive hetero-oligomers were also present for these structurally related receptors.

We have previously demonstrated that receptor-receptor interactions between different CCK receptors are not due to random collisions between luciferase and yellow fluorescent proteins but rather that they reflect more specific interactions between the receptors themselves (Cheng et al., 2003). Here, we performed analogous studies to examine the specificity of BRET signals observed in the cases of VPAC receptor

homo- and hetero-oligomers. Figure 6 illustrates BRET ratios from cells coexpressing homo- or hetero-oligomeric BRET pairs of tagged receptors along with a third untagged similar or structurally distinct receptor. As described above, significant BRET signals were observed from all BRET combinations of tagged VPAC1, VPAC2, or secretin receptors. Introduction of untagged VPAC1, VPAC2, or secretin receptors significantly reduced the BRET signals of VPAC homo- and hetero-oligomers (Fig. 6A). Likewise, the BRET signals from VPAC/secretin receptor hetero-oligomers were competitively reduced with untagged VPAC1, VPAC2, or secretin receptors (Fig. 6B). Of note, in none of these cases did coexpression with structurally unrelated, nonfluorescent, fully functional cholecystokinin receptors result in any change in the BRET signal, thus confirming the structural specificity of these interactions.

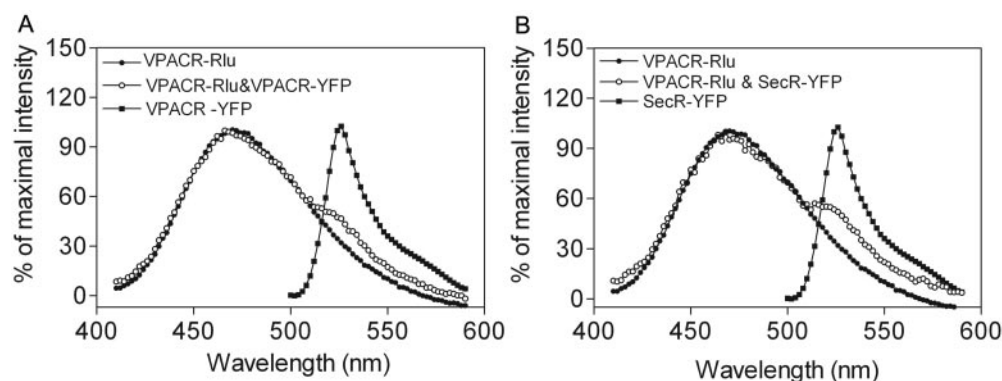
Agonist occupation has been reported to have a variety of effects on the oligomerization of G protein-coupled receptors. Figure 7 shows the results of a series of BRET experiments in which the oligomerization of various receptors was measured in the presence of increasing concentrations of vasoactive intestinal polypeptide and secretin. Agonist exposure significantly ( $p < 0.05$ ) reduced, in a dose-dependent manner, the BRET signal observed for VPAC1 and VPAC2 receptor homo-oligomers (Fig. 7A). That is to say, treatment of intact cells with 10 nM vasoactive intestinal polypeptide reduced the BRET ratio by nearly half, and higher concentrations further modulated homo-oligomeric VPAC1 and VPAC2 receptor interactions. Conversely, treatment of cells with secretin had no effect on the BRET signals attributable to secretin receptor homo-oligomers (Fig. 7A); this finding supports our previous observations (Ding et al., 2002). Vasoactive intestinal polypeptide treatment also significantly reduced the BRET signals for VPAC1 and VPAC2 hetero-oligomers in a concentration-dependent manner, but agonist treatment (vasoactive intestinal polypeptide or secretin) had no effect on either of the VPAC/secretin receptor hetero-oligomers (Fig. 7B). Similar treatments of the latter with both vasoactive intestinal polypeptide and secretin together had no effect on the complexes. Figure 8 illustrates the BRET signal quantification after treatment of receptor-expressing cells with PG97-269, a competitive antagonist for VPAC1 receptors.

TABLE 1

Characterization of tagged VPAC constructs used in BRET studies  
The data shown represent the means  $\pm$  S.E.M. from three to five independent experiments performed in duplicate. Values in parentheses are in nanomolar concentration.

Construct	VIP Binding (pIC <sub>50</sub> )	VIP Activity (pEC <sub>50</sub> )
WT-VPAC1R	8.50 $\pm$ 0.08 (3.16)	9.60 $\pm$ 0.22 (0.25)
VPAC1R-Rlu	9.05 $\pm$ 0.08 (0.89)	9.59 $\pm$ 0.19 (0.26)
VPAC1R-YFP	9.15 $\pm$ 0.07 (0.71)	9.57 $\pm$ 0.14 (0.26)
WT-VPAC2R	8.19 $\pm$ 0.10 (6.49)	8.64 $\pm$ 0.12 (2.32)
VPAC2R-Rlu	8.17 $\pm$ 0.13 (6.82)	9.13 $\pm$ 0.16 (0.74)
VPAC2R-YFP	8.33 $\pm$ 0.19 (4.65)	8.54 $\pm$ 0.25 (2.86)

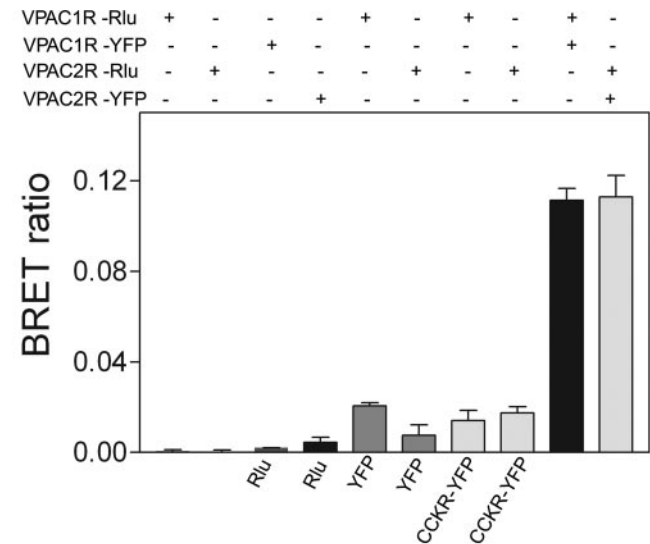
p, negative log molar.



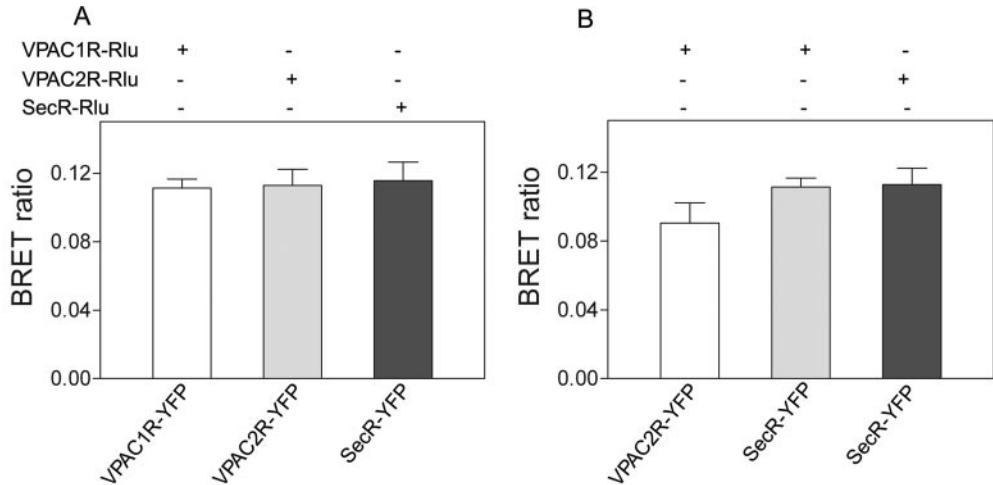
**Fig. 3.** VPAC receptor BRET. Shown are the representative bioluminescence and fluorescence emission curves for COS cells transfected with tagged VPAC receptors or secretin receptor separately or together, as indicated. Rlu and YFP were chosen as BRET pair donor and acceptor, respectively, with each of these attached at the carboxyl terminus of the receptors. The bioluminescence curves were measured with peak intensity at 475 nm and were excited by exposing the cells to coelenterazine h. The YFP fluorescence emission was measured by exciting the cells at 480 nm and recording emission peaks at 525 nm.

This ligand had no effect on the BRET signals attributed to either homo- or hetero-oligomers of VPAC1 and VPAC2 receptors.

One limitation of BRET studies is that they do not distinguish between oligomers that exist at the plasma membrane and those present intracellularly, within the biosynthetic cascade. For this reason, we localized VPAC and secretin receptor oligomers in fixed cells via morphologic FRET analyses of CFP- and YFP-tagged receptors. Figure 9 shows a series of epifluorescence micrographs that were used to evaluate the spectral sensitivities and limitations of the filter cubes used for FRET data collection (see *Materials and Methods*). Images of cells expressing a tandem CFP-YFP fusion protein served as a positive control, whereas images of cells



**Fig. 4.** VPAC receptor BRET controls. Shown are the BRET signals obtained from a series of control experiments performed in cells expressing different combinations of receptor constructs, as indicated. The first eight columns represent the BRET signals obtained with only a single fluorescent VPAC receptor construct or with this paired with a soluble fluorescent partner or with a tagged structurally distinct CCK receptor construct. The last two columns represent the BRET signals obtained when the tagged donor and acceptor VPAC receptors were coexpressed together, as indicated. Data are represented as means  $\pm$  S.E.M. of three independent experiments. A constant amount of DNA (3  $\mu$ g/10-cm plate) was maintained throughout the transfection procedure to get similar levels of expression for each of the constructs used.



**Fig. 5.** Homo- and hetero-oligomerization of VPAC receptors. Shown are the BRET signals associated with receptor homo-oligomerization (A) and hetero-oligomerization (B) when noted constructs were expressed in COS cells. Data represent means  $\pm$  S.E.M. from four independent experiments.

coexpressing soluble CFP and YFP proteins separately provided negative controls. Robust, background and bleed-through-corrected FRET signals were observed in cells expressing the CFP-YFP fusion protein (Fig. 9A). Cross-talk into the FRET channel was removed consistently via sensitized emission calculations after routine quantification of images of cells expressing CFP (Fig. 9B) or YFP (Fig. 9C) alone. Such rigorous corrections were confirmed by the lack of signal in the corrected FRET channel when CFP and YFP were coexpressed as separate proteins within the cytosol (Fig. 9D). The results of these control studies were similar to those reported by others using this sensitized emission method for FRET imaging (Canals et al., 2004; Carrillo et al., 2004).

Shown in Fig. 10 are the results of morphologic FRET localization of pairs of CFP- and YFP-tagged receptors coexpressed in COS cells. Similar to the data presented in Fig. 1 for YFP-tagged receptors, all of the fluorescent protein-tagged VPAC1, VPAC2, and secretin receptors were localized in the CFP and YFP channels within both the plasma membrane and biosynthetic endoplasmic reticulum and Golgi compartments when coexpressed as homo-oligomers (Fig. 10, A–C). In corrected FRET images, these homo-oligomers were detected in the same cell surface and subcellular compartments. Although sometimes difficult to detect in epifluorescence images of adherent COS cells, these fluorescence patterns were reproducible despite minor variations in expression levels and cell morphology (as judged qualitatively by differences in fluorescence intensity; data not shown). These illustrations of cell surface-localized receptor oligomers are consistent with the observed vasoactive intestinal polypeptide-induced modulation of BRET signals in intact cells. Furthermore, FRET localization of oligomers in the endoplasmic reticulum and Golgi suggests that these complexes form early in the biosynthetic pathway. We also explored the subcellular localizations of hetero-oligomeric receptors and found that VPAC1/VPAC2 receptor hetero-oligomers were present similarly on both the plasma membrane as well as intracellularly (Fig. 10D). In contrast, coexpression of VPAC1R or VPAC2R with secretin receptor seemed to block the sorting of the CFP-tagged VPAC member, whereas the YFP-tagged secretin member sorted normally to the plasma membrane (Fig. 10, E and F). It is noteworthy that these hetero-oligomers involving VPAC1R/



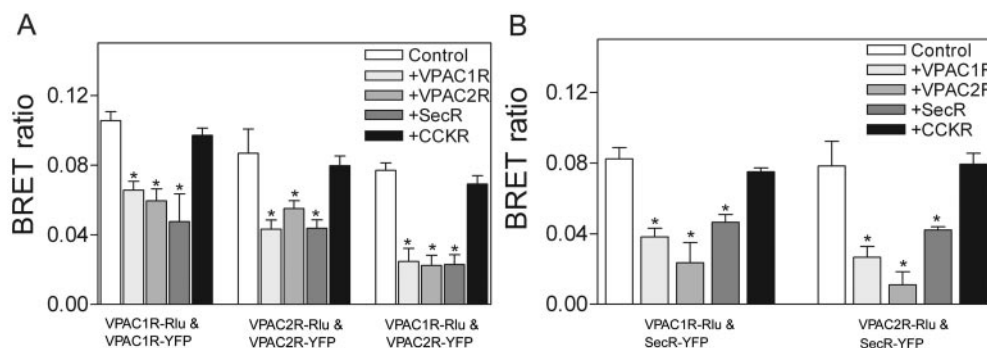
SecR and VPAC2R/SecR were present exclusively within intracellular biosynthetic compartments, without significant localization to the cell surface.

## Discussion

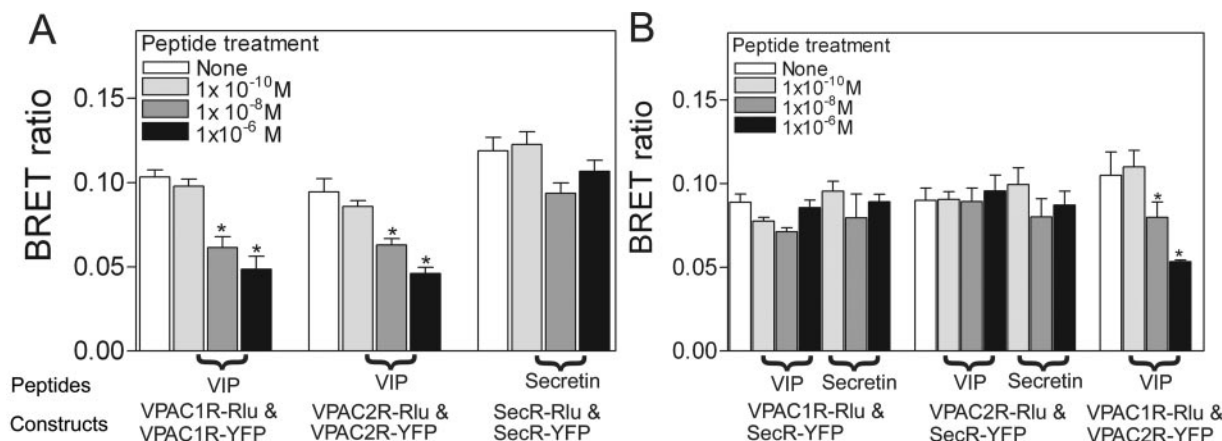
Association of cell surface receptors with themselves, with other structurally related receptors, and with structurally distinct molecules provides a broad repertoire of potential regulatory processes that can be used by a cell. There are now numerous examples of such processes influencing the specificity and selectivity of receptors, changing the sensitivity of the system, augmenting or reducing signaling or desensitization, and even having no apparent effect (Cvejic and Devi, 1997; George et al., 2000; Cheng et al., 2003; Terrillon et al., 2003; Breit et al., 2004). For members of the G protein-coupled receptor superfamily, the ability to oligomerize and the functional significance of this oligomerization have been most extensively studied for family A G protein-coupled receptors (Angers et al., 2000; Rocheville et al., 2000; Ayoub et al., 2002). This has recently been physiologically demonstrated for rhodopsin dimers in murine rod outer segment membranes using atomic force microscopy (Fotiadis et al., 2003). Although there are reported examples of oligomerization of family B

G protein-coupled receptors (Ding et al., 2002; Seck et al., 2003), the evidence is still limited, and no rules have been developed for determining which receptors are able to associate, the functional implications of those complexes, or the effect of ligand occupation on the complexes. The observations in the current report provide an important expansion of our understanding of the processes of oligomerization of family B G protein-coupled receptors and demonstrate that there is diversity of responses to agonist binding for even the most closely related receptors.

In this work, we have used BRET analyses in living cells and morphologic FRET imaging to demonstrate that a group of structurally closely related family B G protein-coupled receptors, specifically the secretin, VPAC1, and VPAC2 receptors, constitutively form homo-oligomeric complexes. In addition, they are capable of interacting with each other to constitutively form hetero-oligomeric complexes in cells in which they are coexpressed. Of particular interest, the effect of ligand occupation on these complexes varies with the character of the ligand and with the specific receptor involved. Unlike the secretin receptor, with homo-oligomeric complexes that are unaffected by ligand binding (Ding et al., 2002; this study), homo-oligomeric complexes of VPAC1 and VPAC2 receptors were modulated by agonist peptide binding.

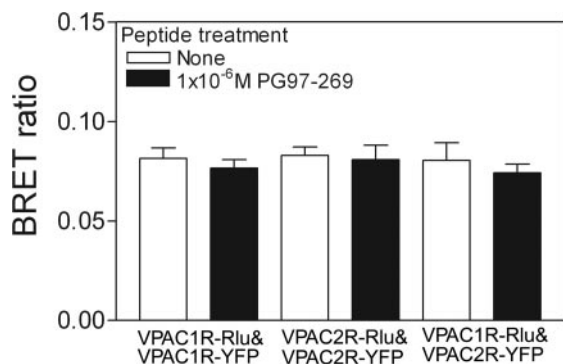


**Fig. 6.** Specificity of VPAC receptor BRET signal. Shown are the quantification of BRET signals acquired from a series of experiments after the COS cells were transiently transfected in the presence and absence of structurally similar untagged receptor constructs or structurally different receptors, as indicated. VPAC receptor homo-oligomers (A) and VPAC receptor hetero-oligomers (B). Data represent means  $\pm$  S.E.M. from three independent experiments. \*,  $p < 0.05$  compared with the BRET signal derived from cells expressing tagged receptors without untagged competing receptors.

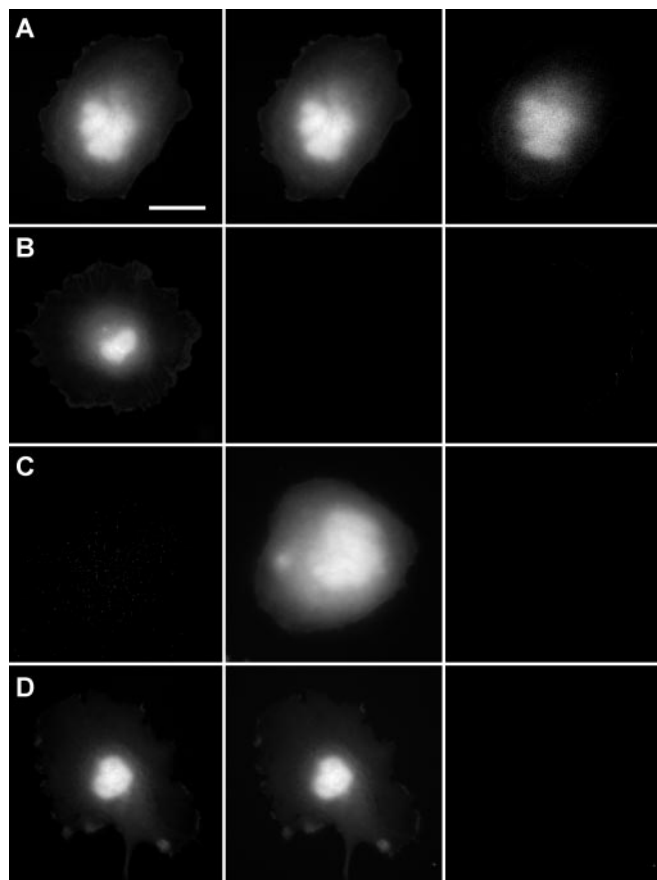


**Fig. 7.** Agonist binding and VPAC receptor BRET signal. A, specific effects of vasoactive intestinal polypeptide stimulation on the BRET ratios of VPAC1 and VPAC2 homo-oligomers or secretin homo-oligomers. B, effect of vasoactive intestinal polypeptide and secretin on the BRET ratios of hetero-oligomers of VPAC1 or VPAC2 receptors with secretin receptors. There was a significant concentration-dependent reduction in the BRET signal for homo- and hetero-oligomers that included both VPAC receptors, and there was no change observed for homo- and hetero-oligomers that included secretin receptors. Data represent means  $\pm$  S.E.M. from three independent experiments. \*,  $p < 0.05$  compared with BRET signal from the same cells without agonist treatment.

In contrast, antagonist binding did not affect VPAC receptor complexes. In addition, hetero-oligomeric complexes including the VPAC1 and VPAC2 receptors were modulated by vasoactive intestinal polypeptide binding. All of these conditions resulted in normal trafficking of the receptors to the cell

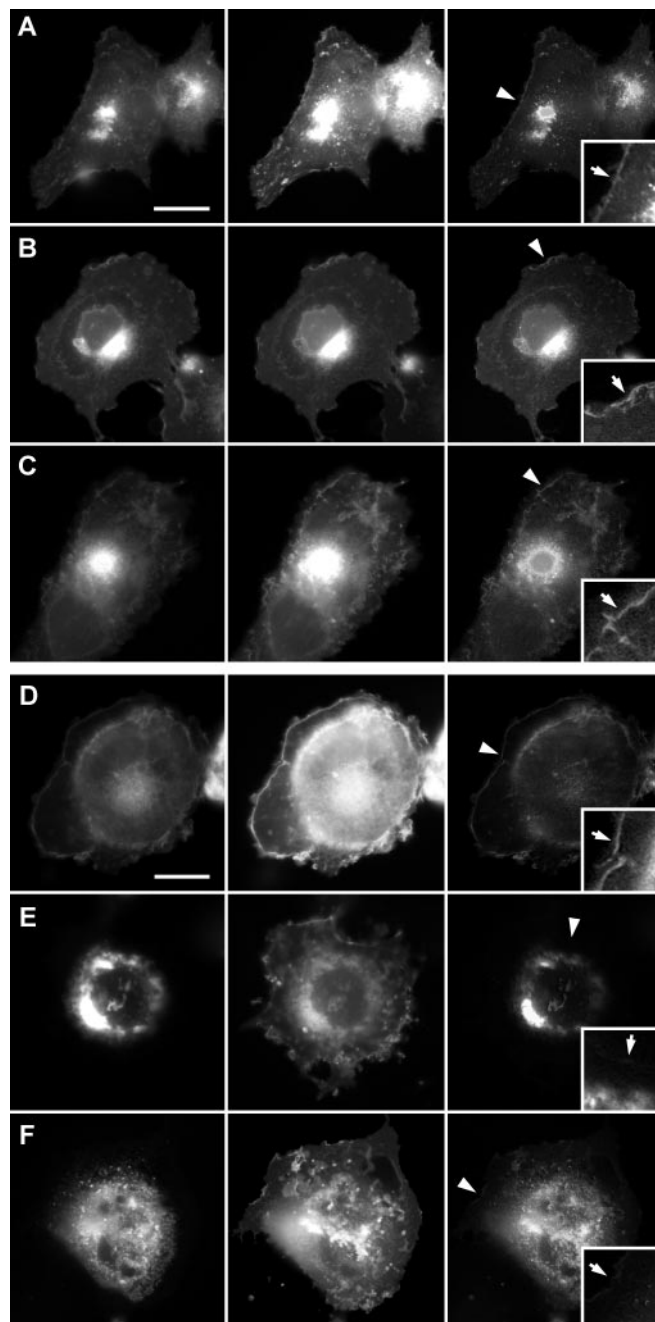


**Fig. 8.** Effect of antagonist on VPAC receptor BRET signal. Shown are the BRET signals derived with specific effect of VPAC antagonist on VPAC homo- and hetero-oligomers. There were no changes in the BRET signals obtained from VPAC receptor homo- or hetero-oligomers exposed to the VPAC1 receptor-specific antagonist PG97-269. Data represent means  $\pm$  S.E.M. from three independent experiments.



**Fig. 9.** Fluorescence microscopy controls for morphologic FRET analyses. Shown are representative epifluorescence micrographs of formaldehyde-fixed COS cells expressing a CFP-YFP fusion protein (A), CFP only (B), YFP only (C), or coexpressing CFP and YFP (D). Images in the left, middle, and right columns represent CFP, YFP, and FRET (corrected) signals, respectively. These experiments provided the positive and negative controls that were used to determine the bleed-through coefficients needed for calculating corrected FRET (see *Materials and Methods*). Scale bar in A, 25  $\mu$ m.

surface where the receptor interactions were shown to occur. Of particular note, coexpression of the secretin receptor and either type of VPAC receptor resulted in intracellular trapping of hetero-oligomeric complexes that were unaffected by



**Fig. 10.** Morphologic FRET analyses of VPAC and secretin receptor oligomerization. Shown are representative micrographs of fixed COS cells expressing various combinations of CFP- and YFP-tagged receptors. Images in the left, middle, and right columns represent CFP, YFP, and FRET (corrected) signals, respectively. A to C, images of cells coexpressing VPAC1R-CFP and VPAC1R-YFP (A), VPAC2R-CFP and VPAC2R-YFP (B), or SecR-CFP and SecR-YFP (C). Homo-oligomers of all three receptors were detected via corrected FRET in the biosynthetic cascade and the plasma membrane (arrows in insets). D to F, images of cells coexpressing VPAC1R-CFP and VPAC2R-YFP (D), VPAC1R-CFP and SecR-YFP (E), or VPAC2R-CFP and SecR-YFP (F). Hetero-oligomers of all three receptors also were localized in biosynthetic organelles, but only VPAC1-VPAC2 receptor hetero-oligomers sorted to the plasma membrane (arrows in insets). Scale bars in A and D, 25  $\mu$ m. Insets are 2.5-fold enlargements of the regions marked by arrowheads.



either secretin or by vasoactive intestinal polypeptide exposure. Such trapping could provide a mechanism for dominant negative inhibition of the secretin receptor on vasoactive intestinal polypeptide action on cells expressing both VPAC and secretin receptors.

BRET represents a powerful experimental approach to establish the association of two molecules in a living cell. By including careful and extensive controls in the current report, it is clear that the BRET signal could not be reproduced by coexpression of the Rlu and YFP transfer partners in distinct compartments of a single cell and could not even be reproduced by expression of the transfer partners in a single cellular compartment (i.e., the plasma membrane) when they were attached to structurally unrelated G protein-coupled receptors. Furthermore, the nonfluorescent receptors were able to competitively inhibit the BRET signal only when they were structurally related to the pair of receptors being studied. The structural specificity of the signal in these assays validates our current interpretations of receptor oligomerization.

The modulation of the BRET signal under certain experimental conditions, however, is more difficult to interpret. In the current study, some of the complexes were modulated by binding of agonists but not antagonists, the former having been demonstrated by a dose-dependent decrease in the BRET signal. This may reflect dissociation of the oligomeric complexes, or a conformational change without dissociation that led to increased distance between donor and acceptor or a distinct change in the orientation of the dipoles. In studying the oligomerization of the CCK receptor, we have been able to vary the position of the fluorescent tag without changing the impact of ligand occupation on the BRET signal, supporting true dissociation rather than conformational change within an intact complex (Cheng and Miller, 2001). This approach is more difficult to achieve for secretin family receptors where the structure-activity considerations more rigorously limit the sites within the receptor that can successfully accommodate the fluorescent tag (Dong et al., 2004); the amino terminus of these receptors is particularly difficult to tag successfully.

It was intriguing that neither secretin nor vasoactive intestinal polypeptide had any influence on the BRET signal coming from cells coexpressing both the secretin receptor and either type of VPAC receptor in COS cells. This was clarified when morphologic FRET imaging was applied to the same conditions and demonstrated that receptor trafficking was markedly impaired by such coexpression of these receptor pairs. In every other condition studied, including the coexpression of VPAC1 and VPAC2 receptors, these constructs trafficked normally to the cell surface. When the secretin receptor was expressed with the VPAC receptors, both were largely trapped in the biosynthetic pathway where there was a strong FRET signal demonstrating receptor-receptor interaction, yet there was no significant FRET signal coming from the cell surface. Although it is possible that the different levels of receptor expression and different kinetics of biosynthesis that might occur during natural expression of these receptors in cells may lead to greater cell surface VPAC receptor expression, it is noteworthy that no cell surface VPAC receptor expression was observed despite a high level of receptor protein expression. Under these conditions, there was a strong intracellular FRET signal, suggesting that most

VPAC receptors were indeed hetero-oligomerized with secretin receptors and that this led to their retention within the cell. This also suggests that the hetero-oligomer between VPAC and secretin receptors predominates over homo-oligomeric complexes, at least for the VPAC receptors. In these experiments, secretin receptors could still be observed at the cell surface. This is likely to reflect a more efficient expression and/or trafficking of the secretin receptors over the VPAC receptors under these conditions.

The hetero-oligomerization of family B receptors has significant implications for how we view receptor pharmacology within this receptor class. It is evident that many members of this receptor family, including the VPAC1 receptor, can also hetero-oligomerize with the receptor activity modifying family of proteins (RAMPs) (Christopoulos et al., 2003). To date, such interactions have only been considered in the context of "individual" or homo-oligomeric receptors. As functionally characterized RAMP partners, the calcitonin receptor and calcitonin-like receptor can both exist as homo-dimeric receptors (Seck et al., 2003; Heroux and Bouvier, 2005) in an analogous manner to each of the receptors in the current study, and RAMPs themselves clearly form homodimers (McLatchie et al., 1998; Sexton et al., 2001). Furthermore, we have shown that cotransfection of either VPAC1 or calcitonin-like receptors with RAMPs leads to loss of RAMP homodimer (Udawela et al., 2004), implying an exclusivity of the dimer interface, at least for the RAMPs, but no loss of cell surface expression of the VPAC1 receptor (Christopoulos et al., 2003). The VPAC1 receptor interacts strongly with all three types of RAMPs and most tissues/cells express at least one RAMP (Sexton et al., 2001; Christopoulos et al., 2003; Hay et al., 2006), suggesting that the VPAC1-RAMP interaction may be widespread. In the current study, we demonstrate that coexpression of the secretin receptor with either of the VPAC receptors leads to loss of cell surface expression of the VPAC receptors as well as a lack of cell surface FRET between the secretin receptor and the VPAC receptor. As discussed above, this interaction is likely to lead to dominant negative effects of the secretin receptor on VPAC receptor function when the two are physiologically expressed. These interactions have implications for VPAC1/RAMP heterodimers; secretin receptor coexpression may also lead to competitive loss of functional VPAC1/RAMP dimers, although it is possible that RAMP expression could rescue VPAC1 receptors from dominant negative effects of the secretin receptor. Regardless, the oligomerization of the VPAC1 receptor with RAMPs provides an additional layer of complexity on the biology of this family of receptors with competition of receptor homo-oligomers, receptor hetero-oligomers, and receptor/accessory protein oligomers potentially yielding different functional outcomes.

The interaction of RAMPs with family B receptors may also provide some insight into potential mechanisms for formation of dimeric receptors. Like family B receptors, single transmembrane domain RAMPs contain a large extracellular domain that is structured by conserved disulfide bridges. In the best studied case of RAMP-receptor interaction, that of the calcitonin-like receptor with RAMP1, both the amino-terminal extracellular domain and transmembrane domain of RAMP1 contribute to the interface, and indeed weak, but functional dimerization can occur via the amino-terminal domain alone (Fitzsimmons et al., 2003; Ittner et al., 2005). It

is possible, therefore, that an analogous situation occurs in the formation of receptor homo- and hetero-dimers, although this remains to be empirically determined.

The capacity of VPAC1, VPAC2, and secretin receptors to interact with each other is of direct physiologic and pathophysiologic relevance, because they can be naturally coexpressed on various types of cells, including normal pancreatic acinar cells and cells involved in pathologic states, such as ductular pancreatic carcinoma cells (Estival et al., 1981, 1983; Ding et al., 2002). Furthermore, these receptors can have completely different biologic effects on a given type of cell. It is interesting that vasoactive intestinal polypeptide can be growth-stimulatory, whereas secretin can be growth inhibitory when expressed in the same cellular environment, despite both signaling through a  $G_s$ -coupled adenylate cyclase-cAMP pathway (Gardner et al., 1976; Jensen et al., 1983). This suggests that the receptors also modulate distinct signaling pathways, either through direct coupling to alternative G proteins or through interaction with accessory proteins such as RAMPs (Christopoulos et al., 2003), and the dynamic regulation of such interactions via receptor-receptor complexing is one that also needs to be explored. The observed variations in effects of natural ligand binding on the stability of oligomeric complexes of each of these receptors, with secretin receptor complexes insensitive to secretin exposure and VPAC receptors affected by exposure to vasoactive intestinal polypeptide, could also lead to differential effects on the sensitivity or desensitization of the signaling system. Unfortunately, the study of such complexes in vivo using these techniques is problematic, because it is not yet possible to fluorescently tag endogenous receptors. In the future, new techniques will have to be developed to determine the existence and functional impact of family B G protein-coupled receptor oligomers in naturally occurring cells.

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