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Constitutive Formation of Oligomeric Complexes Between Family B G Protein-coupled VPAC and Secretin Receptors

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Running Title: VPAC and secretin receptor oligomerization

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ABBREVIATIONS: BRET, bioluminescence resonance energy transfer; FRET, fluorescence resonance energy transfer; Rlu, *Renilla* luciferase; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; VPAC1R, type 1 vasoactive intestinal polypeptide receptor; VPAC2R, type 2 vasoactive intestinal polypeptide receptor; SecR, secretin receptor; VIP, vasoactive intestinal polypeptide.

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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* 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 were confirmed by control studies. These constructs bound their natural ligands specifically and saturably, with these agonists 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. Additionally, each of these three receptors formed hetero-oligomers with each other. The VPAC1-VPAC2 hetero-oligomers were modulated by vasoactive intestinal polypeptide binding, while 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 insights into the ability of Family B G protein-coupled receptors to associate with each other in cells.

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INTRODUCTION

Plasma membrane receptors represent a critically important interface between circulating and extracellular hormones and neurotransmitters and the cell's intracellular signaling 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 is also relevant to the heptahelical guanine nucleotide-binding protein (G protein)-coupled receptors both in physiological and pathological settings (Ding et 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 et al., 2003; Cheng and Miller, 2001). These processes have been most extensively examined for Family A G protein-coupled receptors (Angers et al., 2000; Ayoub et al., 2002; Carrillo et al., 2003; Cheng et al., 2003; Rocheville et al., 2000). 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 (Cheng et al., 2003; Ding et al., 2002; Gomes et al., 2002; Kroeger et al., 2001; Latif et al., 2002; McVey et al., 2001; Pfleger and Eidne, 2005; Terrillon et al., 2003). 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 densensitization (Carrillo et al., 2003; George et al., 2000).

While 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

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tail that contains six highly-conserved cysteine residues known to contribute to three highly-conserved disulfide bonds (Lisenbee et al., 2005; Qi et al., 1997). 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. Additionally, 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-dependency of the oligomers, we also performed morphologic fluorescence resonance energy transfer (FRET) to explore cellular compartments in which receptor association was occurring. While 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.

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EXPERIMENTAL PROCEDURES

Materials. Vasoactive intestinal polypeptide was purchased from Auspep (Parkville, VIC, Australia). Bovine serum albumin and isobutyl methylxanthine (IBMX) were from Sigma (St.Louis, MO) and amplified luminescent proximity homogenous assay (ALPHA)-screen cAMP kits were purchased from Perkin Elmer (Boston, MA). Dulbecco's modified Eagle's media (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 (Roskible, Denmark) and Metafectine was from Scientifix (Cheltenham, VIC, Australia). ^{125}I-VIP was prepared with Na-^{125}I (100mCi/ml) (ICN Biochemicals Irvine, CA) using IODO-BEADS (Pierce Chemical Co, Rockville, IL), with the mono-iodinated peptide purified by reversed-phase high performance liquid chromatography.

cDNA Constructs. The *Renilla* 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. Briefly, the Rlu gene was PCR amplified and TA 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^2-N1 vector (Perkin Elmer, Wellesley, MA): Ser^{66}-Gly, Val^{69}-Leu, Ser^{73}-Ala and Thr^{204}-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^2-N1 amino acids Phe^{65}-Leu, Ser^{66}-Thr, Tyr^{67}-Trp, Asn^{147}-Ile, Met^{154}-Thr, and Val^{164}-Ala. cDNA encoding the VPAC1 and VPAC2 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 blunt-end PCR products were cloned into the pENTR/D-TOPO vector using the pENTR Directional TOPO Cloning

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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 recombinase 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 (Clontech) 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 Dulbecco's Modified Eagle's Medium (DMEM) supplemented with either 5% v/v fetal bovine serum (FBS) or 5% v/v Fetal Clone II and maintained at 37 °C in a humidified atmosphere of 5% CO_2. For cAMP and binding assays, cells were seeded one day prior to transfection into either 48 (1 cm^2)-well plates or 6 (8 cm^2)-well plates at ~125,000 cells/well and 1,000,000 cells/well, respectively. The following day, when cells were 90-100% confluent, they were transfected using 0.75 μl/cm^2 of Metafectine and 50-100 ng/cm^2 of 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 hr incubation, an equal volume of DMEM containing 5% FBS was added. Cells to be used in radioligand binding were incubated under growth conditions for a further 36 hrs, while cells used for cAMP measurement were incubated overnight before being serum-starved for an additional 24 hrs. For BRET assays and fluorescence microscopy, cells were plated at a density of 500,000 cells/dish in 10 cm Petri dishes one day prior to transfection. The transfection procedure was performed by the previously-established DEAE-dextran method using a total DNA concentration of 3 μg per 10 cm dish (Cheng and Miller, 2001).

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Measurement of cAMP. Cells were harvested, counted and diluted to 10,000 cells/5 µl in phenol red-free DMEM containing 0.1% w/v bovine serum albumin (BSA) and 1 mM IBMX (stimulation buffer), and incubated for at least 30 min at 37 °C. Agonist dilutions were prepared in stimulation buffer with 5 µl added to white Opti-384 well plates (Perkin Elmer), with each point repeated in triplicate. Following the 30 min cell incubation, 10,000 cells were added per well in a volume of 5 µl. The plates were briefly centrifuged then incubated for 30 min at 37 °C. Agonist-stimulated receptor activity was terminated by the addition of 10 µl lysis buffer (5 nM HEPES, pH 7.4, 0.3% v/v Tween 20, 0.1% w/v BSA). The cAMP level was assayed in the same wells using ALPHA-screen assay kits. Briefly, 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. Following overnight incubation in the dark, the plates were read with an ALPHA-screen protocol on a Perkin Elmer Fusion plate reader (Boston, MA). Data were analyzed using Graphpad Prism 4 software (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 hrs 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 unlabelled peptide. Cells were incubated for 1 hr at 37 °C before being washed with 250 µl PBS, and then solubilized with 250 µl of 0.5 M NaOH. The cell lysate was collected and counted in a Packard γ-counter (75% efficiency) to determine bound radioactivity. Competition-binding data were analyzed via non-linear regression using Graphpad Prism 4.

BRET Studies. Transiently-transfected COS cells were detached from culture flasks 48 hrs post-transfection with a non-enzymatic cell-dissociation solution (Sigma). The cells were washed with Krebs-

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Ringers/HEPES (KRH) solution (25 mM HEPES, pH 7.4, 104 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 2 mM $CaCl_2$, 1 mM KH_2PO_4 plus 0.2% w/v BSA and 0.01% w/v soybean trypsin inhibitor) and then resuspended in KRH to a final concentration of approximately 1,000,000 cells/ml. Where appropriate, aliquots of these cells were treated with various ligands at 37 ^oC for 2 min prior to BRET assays that were performed as we previously described (Cheng and Miller, 2001). Briefly, the cell-permeant *Renilla* luciferase substrate, coelenterazine *h* (Biotium, Hayward, CA), was added to one million cells in a one-ml quartz cuvette to a final concentration of 5 μM. Bioluminescence emissions were monitored immediately in a SPEX FluoroMax-3 spectrofluorometer (SPEX Industries, Edison, NJ) in the spectral range between 400 and 600 nm using wavelength increments of 2 nm and an integration time of 2 sec. 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 and 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)×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 hrs after transfection and seeded to UV-sterilized 25 mm round coverslips in 6-well plates. After culture for an additional 24 to 48 hrs, 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 Vectashield (Vector Laboratories, Burlingame, CA) and then observed and photographed with a Zeiss (Thornwood, NY) LSM 510 confocal microscope configured for YFP fluorescence (excitation, 514 nm argon laser; emission, LP530 filter; pinhole diameter, one airy unit; objective, Plan-Apochromat 63X/1.4NA oil). Morphologic FRET imaging utilized a Zeiss Axiovert 200M microscope equipped with dedicated CFP (excitation 436/20, dichroic 455DCLP, emission 480/40), YFP (excitation 500/20, dichroic Q515LP, emission 535/30), and FRET (excitation 436/20, dichroic 455DCLP, emission 535/30) epifluorescence filter sets (Chroma Technology Corp, Rockingham,

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VT). Digital micrographs were collected separately from all three channels with a Hamamatsu (Bridgewater, NJ) monochromatic ORCA-ER CCD camera. Image acquisition was automated with QED InVivo software v. 2.0 (Media Cybernetics, 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 v. 6.3 (Molecular Devices, Sunnyvale, CA) using the sensitized emission method. Here, corrected FRET is defined as FRETc = FRET – (B × CFP) – (A × 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 threshholded 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 ± 0.01 and A = 0.15 ± 0.01 and represent the means ± S.E.M. for five separate data sets. Micrographs were assembled into figures and adjusted for contrast using Adobe Photoshop v. 7.0 (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.

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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 properly to the plasma membrane. A significant amount of intracellular fluorescence also was observed in the endoplasmic reticulum and golgi cluster, likely as a result 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 biological 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. Previously, we 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 475-480 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

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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 10-100 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. Previously, we used a soluble Rlu-YFP fusion protein construct as a positive control to demonstrate a significant BRET signal (BRET ratio ~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, non-specific 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 due to 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 Figure 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 ratio values of approximately 0.11. There was little or no BRET signal (BRET ratio ~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 ~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,

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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. Additionally, 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 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 earlier, 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). Similarly, 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 non-fluorescent 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

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(Fig 7A). Specifically, 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. 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 employed for FRET data collection (see Experimental Procedures). Images of cells expressing a tandem CFP-YFP fusion protein served as a positive control, whereas images of cells 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

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(Fig 9D). The results of these control studies were similar to what has been reported by others using this sensitized emission method for FRET imaging (Canals et al., 2004; Carrillo et al., 2004).

Shown in Figure 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 Figure 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 10A-C). In corrected FRET images these homo-oligomers were detected in the same cell surface and subcellular compartments. Though 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 appeared to block the sorting of the CFP-tagged VPAC member while the YFP-tagged secretin member sorted normally to the plasma membrane (Fig 10E-F). Interestingly, these hetero-oligomers involving VPAC1R/SecR and VPAC2R/SecR were present exclusively within intracellular biosynthetic compartments, without significant localization to the cell surface.

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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 utilized 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 (Breit et al., 2004; Cheng et al., 2003; George et al., 2000; Cvejic and Devi, 1997; Terrillon et al., 2003). For members of the G protein-coupled receptor superfamily, the ability to oligomerize and the functional significance of this have been most extensively studied for Family A G protein-coupled receptors (Angers et al., 2000; Ayoub et al., 2002; Rocheville et al., 2000). This has recently been physiologically demonstrated for rhodopsin dimers in murine rod outer segment membranes using atomic force microscopy (Fotiadis et al., 2003). While 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 quite 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 utilized 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. Additionally, 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.,

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2002 and this study), homo-oligomeric complexes of VPAC1 and VPAC2 receptors were modulated by agonist peptide binding. In contrast, antagonist binding did not affect VPAC receptor complexes. Additionally, 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 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 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. Further, the non-fluorescent 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

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orientation of the dipoles. Previously, in studying the oligomerization of the CCK receptor, we were 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-oligomerised 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 likely reflects a more efficient expression and/or trafficking of the secretin receptors over the VPAC receptors under these conditions.

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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 et al., 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 co-transfection 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 (Christopoulos et al., 2003; Sexton et al., 2001; Hay et al., 2005), 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.

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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 physiological and pathophysiological relevance, since 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 (Ding et al., 2002; Estival et al., 1981; Estival et al., 1983). Further, these receptors can have completely different biological effects on a given type of cell. It is of interest that vasoactive intestinal polypeptide can be growth-stimulatory, while 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, since it is not yet possible to fluorescently tag endogenous

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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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FOOTNOTES

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FIGURE LEGENDS

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. Bar in (A) = 25 μm.

Fig 2. Vasoactive intestinal polypeptide binding and biological activity at receptor constructs. Shown are the vasoactive intestinal polypeptide competition-binding curves (*left panel*s) and vasoactive intestinal polypeptide-stimulated cAMP responses (*right panels*) 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 panels*), or cells expressing wild type (WT) VPAC2 receptor or VPAC2 receptor constructs that include carboxyl-terminal fusions in-frame with Rlu or YFP (*bottom panels*). Binding data for each construct were normalized relative to control where no competing unlabeled vasoactive intestinal polpeptide was present, subtracting non-specific binding from both [$(B\text{-}N/B_o\text{-}N) \times 100$]. Specific binding at the VPAC1 receptor represented 85-90% 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 ± S.E.M. of 3 to 4 independent assays performed in duplicate. B, ^{125}I-VIP bound; B_o, binding in the absence of competing ligand; and N, non-specific binding.

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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.

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 *bars* 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 *bars* represent the BRET signals obtained when the tagged donor and acceptor VPAC receptors were coexpressed together, as indicated. Data are represented as means ± S.E.M. of three independent experiments. A constant amount of DNA (3 μg/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 ± S.E.M. from four independent experiments.

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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) VPAC receptor hetero-oligomers (B). Data represent means ± S.E.M. from three independent experiments. * $p < 0.05$ as compared to the BRET signal derived from cells expressing tagged receptors without untagged competing receptors.

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

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 ± S.E.M. from three independent experiments.

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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 Experimental Procedures). Bar in (A) = 25 μm.

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-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-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). Bars in (A, D) = 25 μm. Insets are 2.5-fold enlargements of the regions marked by arrowheads.

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TABLE 1

Characterization of tagged VPAC constructs used in BRET studies

The data shown represent the means ± S.E.M. from three to five independent experiments performed in duplicate. *p*, negative log molar.

Constructs	VIP binding pIC_{50}	VIP activity pEC_{50}
WT-VPAC1R	8.50 ± 0.08 (3.16 nM)	9.60 ± 0.22 (0.25 nM)
VPAC1R-Rlu	9.05 ± 0.08 (0.89 nM)	9.59 ± 0.19 (0.26 nM)
VPAC1R-YFP	9.15 ± 0.07 (0.71 nM)	9.57 ± 0.14 (0.26 nM)
WT-VPAC2R	8.19 ± 0.10 (6.49 nM)	8.64 ± 0.12 (2.32 nM)
VPAC2R-Rlu	8.17 ± 0.13 (6.82 nM)	9.13 ± 0.16 (0.74 nM)
VPAC2R-YFP	8.33 ± 0.19 (4.65 nM)	8.54 ± 0.25 (2.86 nM)

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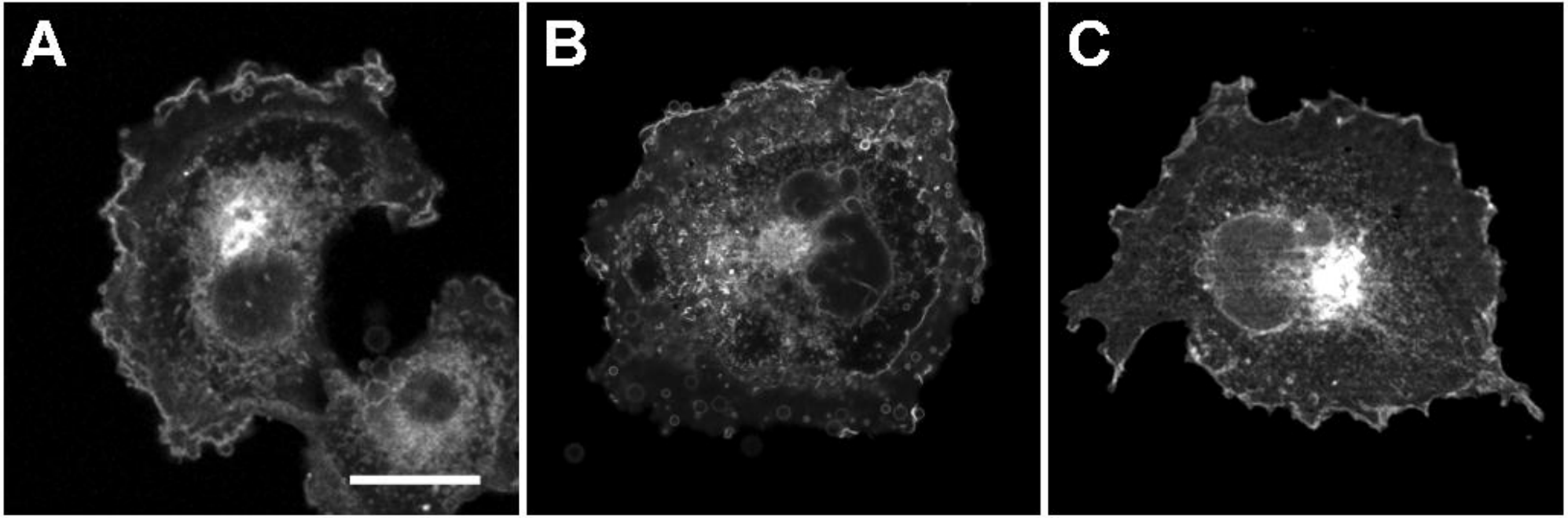

Figure 1

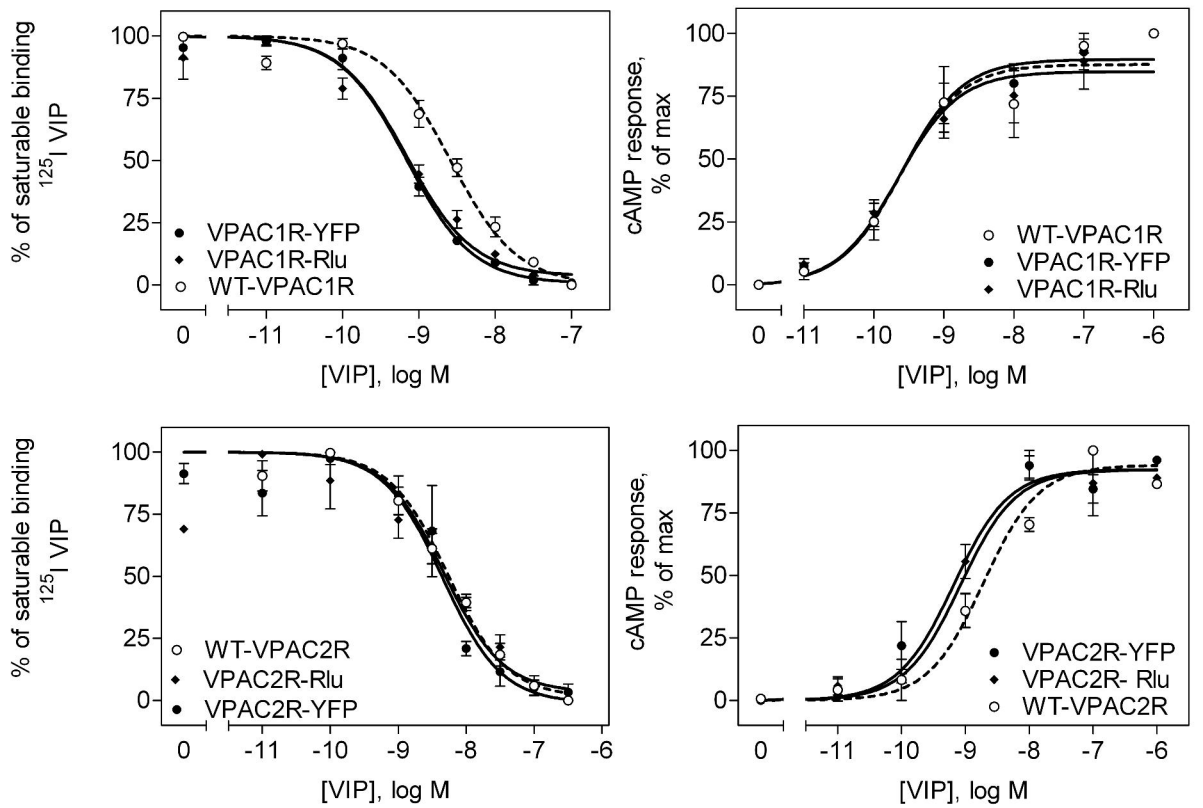
% of saturable binding
125I VIP
100
75
50
25
0
VPAC1R-YFP
VPAC1R-Rlu
WT-VPAC1R
0
-11
-10
-9
-8
-7
[VIP], log M
cAMP response,
% of max
WT-VPAC1R
VPAC1R-YFP
VPAC1R-Rlu
-6
WT-VPAC2R
VPAC2R-Rlu
VPAC2R-YFP
VPAC2R-YFP
VPAC2R- Rlu
WT-VPAC2R

Figure 2

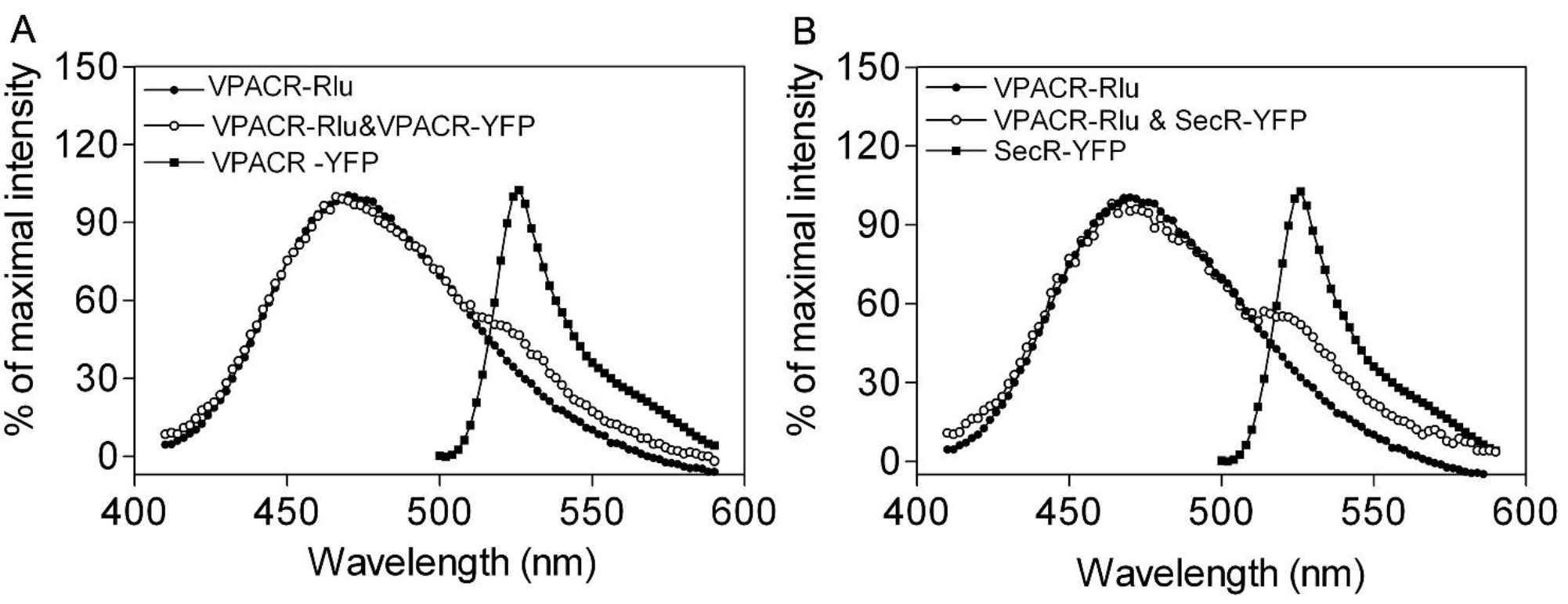
A
% of maximal intensity
150
120
90
60
30
0
VPACR-Rlu
VPACR-Rlu&VPACR-YFP
VPACR -YFP
400
450
500
550
600
Wavelength (nm)
B
% of maximal intensity
150
120
90
60
30
0
VPACR-Rlu
VPACR-Rlu & SecR-YFP
SecR-YFP
400
450
500
550
600
Wavelength (nm)

Figure 3

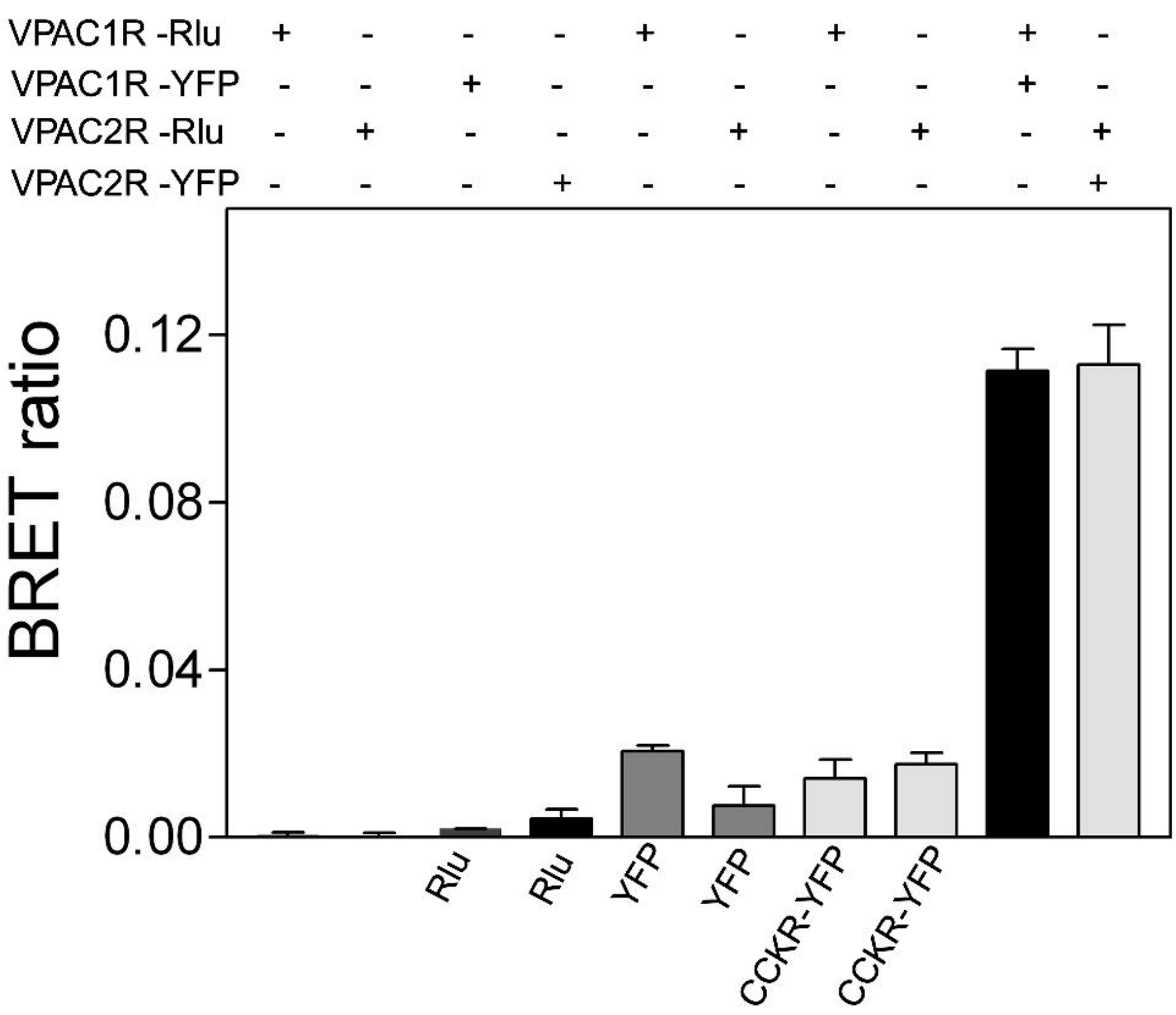
VPAC1R -Rlu + - - - + - + - + -
VPAC1R -YFP - - + - - - - - + -
VPAC2R -Rlu - + - - - + - + - +
VPAC2R -YFP - - - + - - - - - +
BRET ratio
0.12
0.08
0.04
0.00
Rlu
Rlu
YFP
YFP
CCKR-YFP
CCKR-YFP

Figure 4

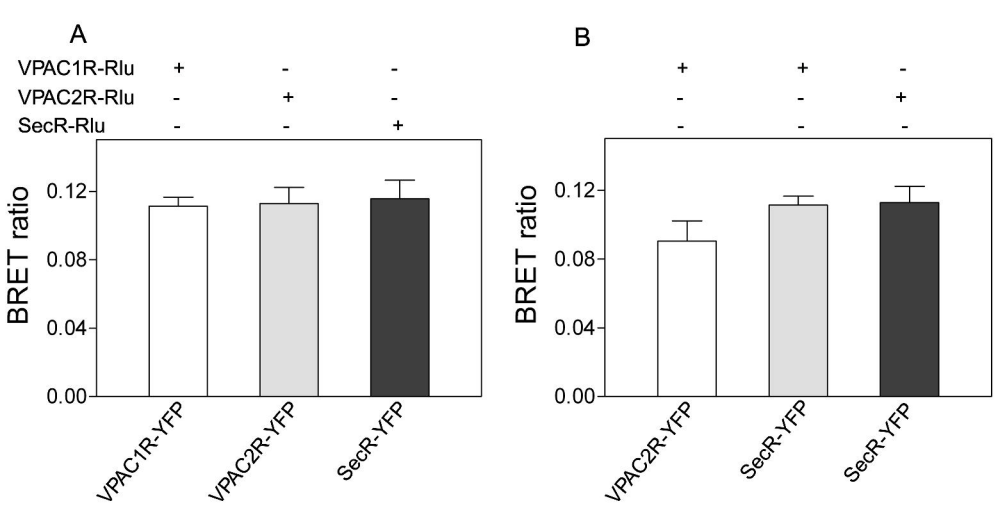
A
VPAC1R-Rlu + - -
VPAC2R-Rlu - + -
SecR-Rlu - - +
BRET ratio
0.12
0.08
0.04
0.00
VPAC1R-YFP
VPAC2R-YFP
SecR-YFP
B
+ + -
- - +
- -
BRET ratio
0.12
0.08
0.04
0.00
VPAC2R-YFP
SecR-YFP
SecR-YFP

Figure 5

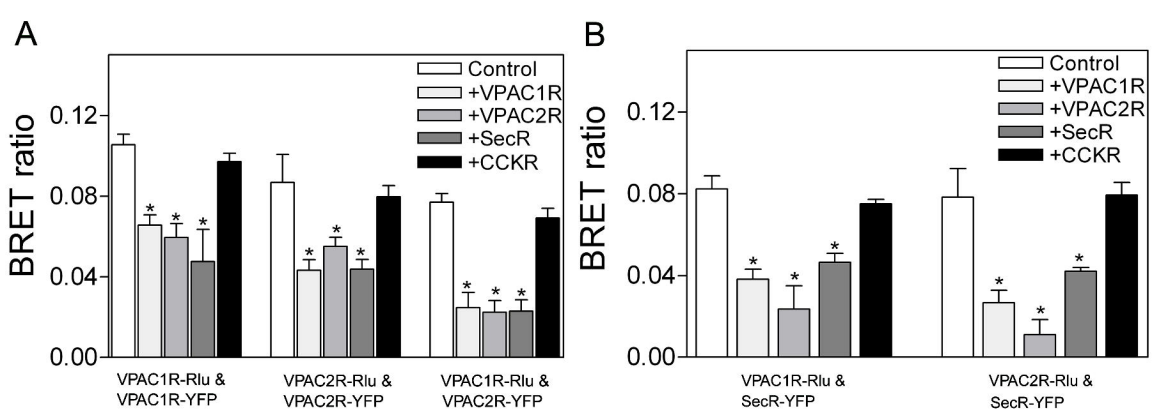
A
BRET ratio
0.00
0.04
0.08
0.12
Control
+VPAC1R
+VPAC2R
+SecR
+CCKR
VPAC1R-Rlu &
VPAC1R-YFP
VPAC2R-Rlu &
VPAC2R-YFP
VPAC1R-Rlu &
VPAC2R-YFP
B
BRET ratio
0.00
0.04
0.08
0.12
Control
+VPAC1R
+VPAC2R
+SecR
+CCKR
VPAC1R-Rlu &
SecR-YFP
VPAC2R-Rlu &
SecR-YFP

Figure 6

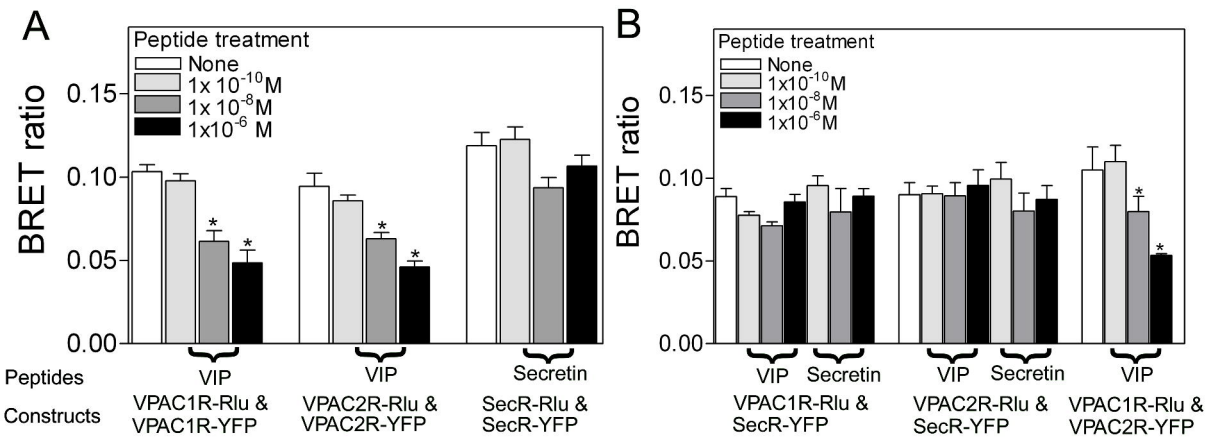
A
Peptide treatment
None
1x 10-10M
1x 10-8M
1x10-6 M
BRET ratio
0.15
0.10
0.05
0.00
*
*
*
*
Peptides
VIP
VIP
Secretin
Constructs
VPAC1R-Rlu &
VPAC1R-YFP
VPAC2R-Rlu &
VPAC2R-YFP
SecR-Rlu &
SecR-YFP
B
Peptide treatment
None
1x10-10M
1x10-8M
1x10-6M
BRET ratio
0.15
0.10
0.05
0.00
*
*
VIP
Secretin
VIP
Secretin
VIP
VPAC1R-Rlu &
SecR-YFP
VPAC2R-Rlu &
SecR-YFP
VPAC1R-Rlu &
VPAC2R-YFP

Figure 7

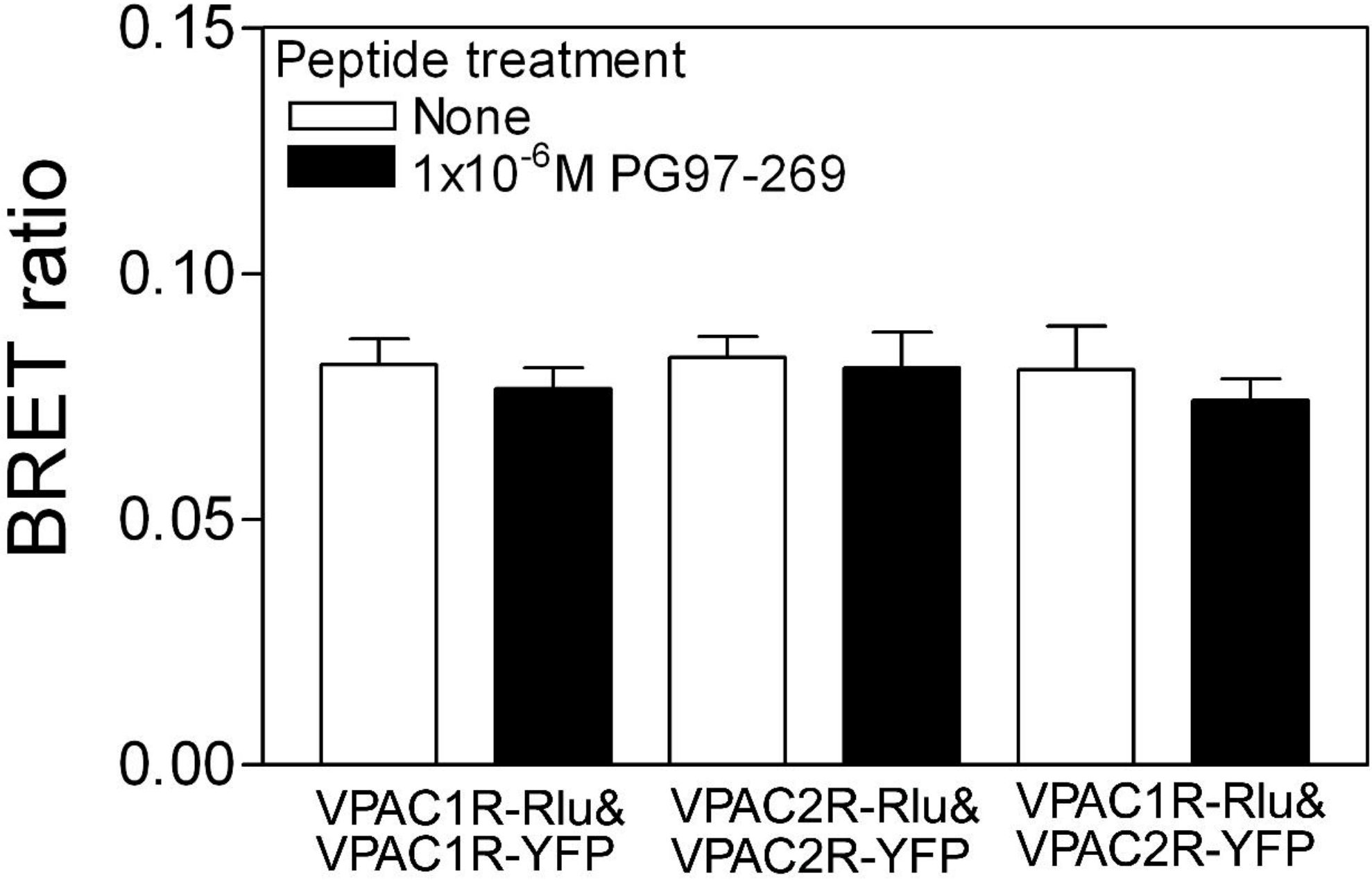
0.15
0.10
0.05
0.00
BRET ratio
Peptide treatment
None
1x10-6M PG97-269
VPAC1R-Rlu&
VPAC1R-YFP
VPAC2R-Rlu&
VPAC2R-YFP
VPAC1R-Rlu&
VPAC2R-YFP

Figure 8

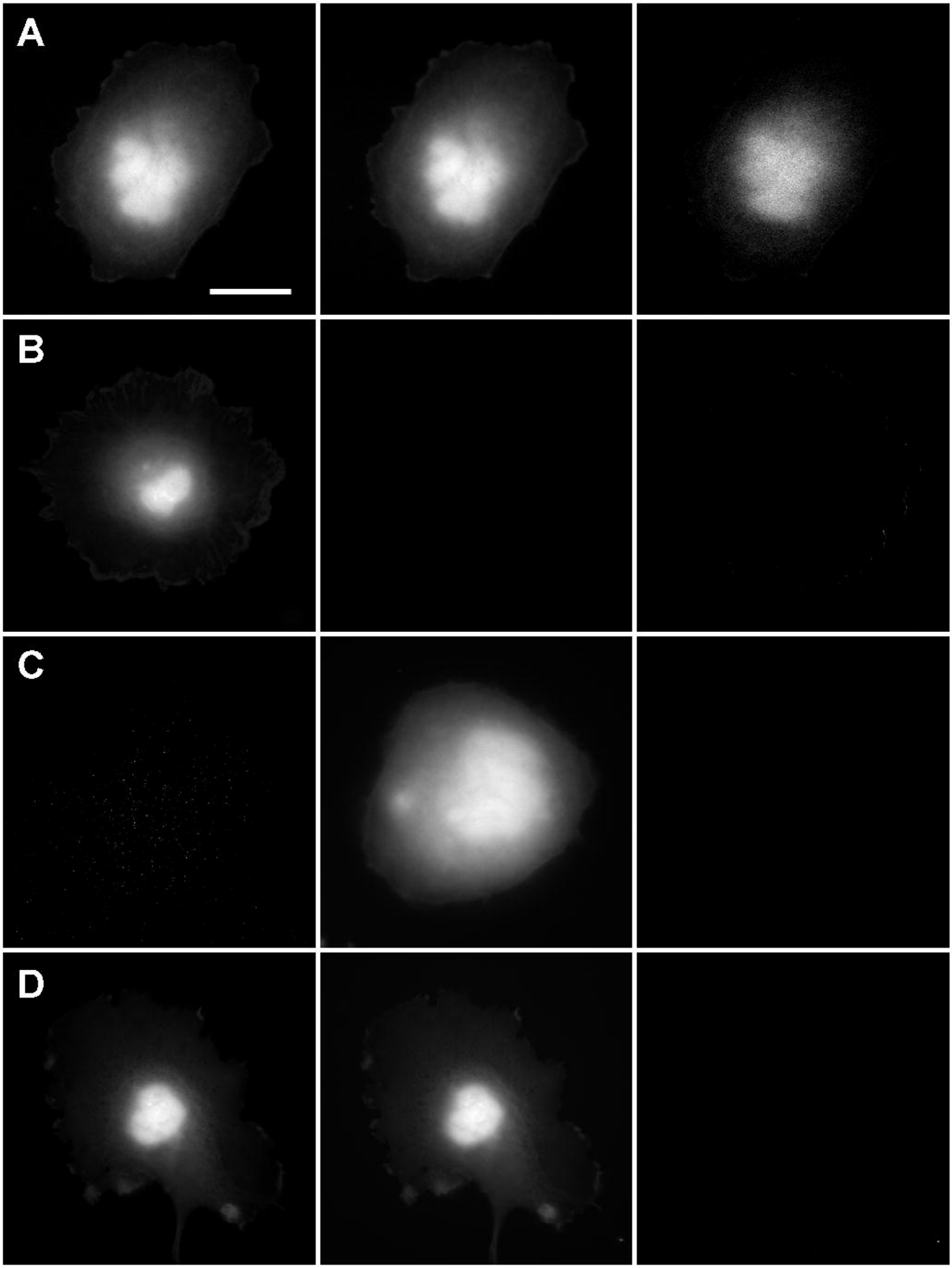

Figure 9

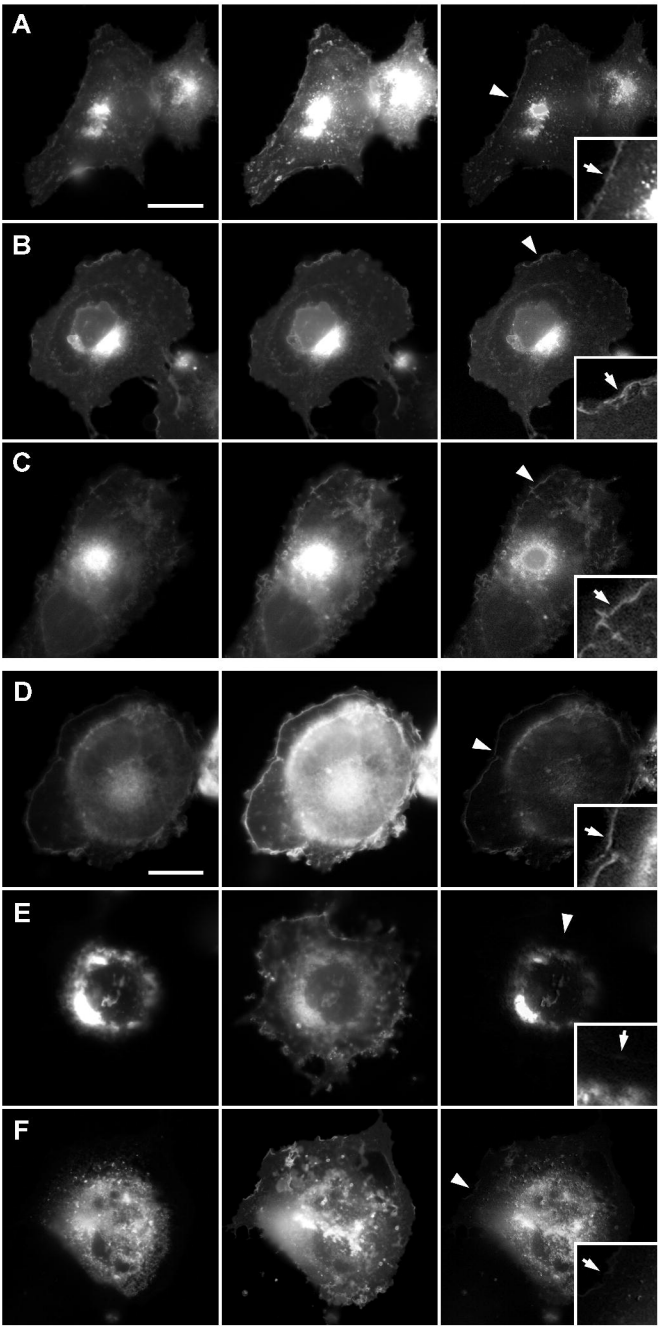

Figure 10