G Protein-coupled Receptors Form Stable Complexes with Inwardly Rectifying Potassium Channels and Adenylyl Cyclase*

Natalie Lavin†‡§, Nathalie Ethier†§, James N. Oak‡, Lin Pei‡, Fang Liu‡, Phan Trieu†, R. Victor Rebois***, Michel Bouvier†‡§§, Terence E. Hébert†§§§, and Hubert H. M. Van Tol†¶¶

From the †Centre for Addiction and Mental Health, Departments of Pharmacology, and the Department of Psychiatry, Institute of Medical Science, University of Toronto, Ontario M5T 1R8, Canada, the ‡Institut de Cardiologie de Montréal, Montréal, Quebec H1T 1C8, Canada, the §§NINDS, National Institutes of Health, Bethesda, Maryland 20892-4449, and the ¶¶Département de biochimie, Université de Montréal, Montréal, Quebec H3C 3J7, Canada

A large number of studies have demonstrated co-purification or co-immunoprecipitation of effectors with G proteins. We have begun to look for the presence of effector molecules in these receptor complexes. Co-expression of different channel and receptor permutations in COS-7 and HEK 293 cells in combination with co-immunoprecipitation experiments established that the dopamine D3 and D4, and β2-adrenergic receptors (β2-AR) form stable complexes with Kir3 channels. The D3/Kir3 and D4/Kir3 receptor/Kir3 interaction does not occur when the channel and receptor are expressed separately and mixed prior to immunoprecipitation, indicating that the interaction is not an artifact of the experimental protocol and reflects a biosynthetic event. The observed complexes are stable in that they are not disrupted by receptor activation or modulation of G protein α subunit function. However, using a peptide that binds Gβγ (βARKct), we show that Gβγ is critical for dopamine receptor-Kir3 complex formation, but not for maintenance of the complex. We also provide evidence that Kir3 channels and another effector, adenylyl cyclase, are stably associated with the β2-adrenergic receptor and can be co-immunoprecipitated by anti-receptor antibodies. Using bioluminescence resonance energy transfer, we have shown that in living cells under physiological conditions, β2AR interacts directly with Kir3.1/3.4 and Kir3.1/3.2c heterotetramers as well as with adenylyl cyclase. All of these interactions are stable in the presence of receptor agonists, suggesting that these signaling complexes persist during signal transduction. In addition, we provide evidence that the receptor-effector complexes are also found in vivo. The observation that several G protein-coupled receptors form stable complexes with their effectors suggests that this arrangement might be a general feature of G protein-coupled signal transduction.

Heterotrimeric (αβγ) guanine nucleotide-binding proteins (G proteins) are the transducers that convey information from agonist-occupied receptors to a variety of effector proteins. Activation of G proteins in detergent-containing solutions results in dissociation of the α subunit (Ga) from the βγ heterodimer (Gβγ). Depending upon the effector that is being regulated, the information is conveyed by Ga and/or Gβγ. Receptors that couple to G proteins (GPCRs) number in the hundreds, and have in common seven α-helical transmembrane domains. Most cells have an assortment of GPCRs, G proteins, and effectors, and in general, signal transduction has been thought of as occurring by a process involving random collisions between these different signaling components as they move about independently of one another in the lipid bilayer. Thus, GPCR-mediated activation of a G protein would be expected to produce Ga and Gβγ with the potential to regulate the activity of multiple effectors. However, signal transduction in vivo most often results in the regulation of a select effector. Recent data, as well as many reports from the early literature on G protein-mediated signaling offer a reason for why expectation does not agree with observation. A number of studies have demonstrated stable interactions between GPCRs and G proteins (see Ref. 1 and 2, for review), between G proteins and effectors (3–5), and between Ga and Gβγ of activated G proteins (4, 6, 7). These data raise the possibility that GPCRs, G proteins, and effectors can form stable complexes that persist during signal transduction in vivo. Such metastable signaling complexes would explain the rapidity and specificity observed during signaling in the intact cell. Indeed, the possibility that GPCRs can form a complex with their effectors was recently shown for the β2-adrenergic receptors (β2AR) and L-type Ca2+ channels (Ca1.2) and adenylyl cyclase (8). This receptor-channel complex also contained heterotrimeric G proteins and the modulatory proteins, protein kinase A and protein phosphatase 2A. Whether GPCRs and other effectors, such as Kir3 channels, are part of stable or transient complexes in which the different components of signaling pathways interact with each other is as of yet unknown. Size exclusion chromatography of purified cardiac I_{KCh} channels (Kir3.1-Kir3.4) identified a complex of 480–520 kDa (9), which would allow for a tetrameric Kir3.1-Kir3.4 structure associated with an equivalent number of muscarinic M2 receptors. Assembly of the GPCR into effector com-

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§ Both authors contributed equally to the results of this article.

†† Canada Research Chair in Molecular and Cellular Pharmacology.

§§ Canada Research Chair in Neurobiology. To whom correspondence should be addressed: Laboratory of Molecular Neurobiology, Centre for Addiction and Mental Health, 250 College St., Toronto, Ontario M5T 1R8, Canada. Tel.: 416-979-4661; Fax: 416-979-4663; E-mail: hubert.van.tol@utoronto.ca.

¶¶ Canada Research Chair in Molecular and Cellular Pharmacology.

# Canada Research Chair in Neurobiology. To whom correspondence should be addressed: Laboratory of Molecular Neurobiology, Centre for Addiction and Mental Health, 250 College St., Toronto, Ontario M5T 1R8, Canada. Tel.: 416-979-4661; Fax: 416-979-4663; E-mail: hubert.van.tol@utoronto.ca.

1 The abbreviations used are: GPCR, G protein-coupled receptor; β2AR, β2-adrenergic receptors; BRET, bioluminescence resonance energy transfer; α-MEM, α-minimal essential medium; PTX, pertussis toxin; HA, hemagglutinin; GST, glutathione S-transferase; PBS, phosphate-buffered saline; EGFP, enhanced green fluorescent protein; RLuc, Renilla reniformis luciferase; AC, adenylyl cyclase; βARKct, β-adrenergic receptor kinase carboxyl terminus.
plexes may be important to restrict surface expression to fully and correctly assembled effector complexes, as has been shown for the sulfonylurea receptor-Kir6 channel assembly (10), GABAB1 and -2 receptor heterodimerization (11), and Kir3.1 channel heterotetramerization with Kir3.2 or Kir3.4 (12). In all these examples correct assembly results from the presence of endoplasmic reticulum retention sequences or post-endoplasmic reticulum targeting motifs. Thus, complex formation may also form a means to regulate GPCR/effector signaling. Furthermore, evidence for the inclusion of many additional proteins involved in downstream signaling events has led to the suggestion that complexes as large as 2000 kDa may represent functional units (13). Using a combination of co-purification strategies, Western blotting, and mass spectrometry, at least 77 different proteins were demonstrated to be associated with the N-methyl-D-aspartate receptor in the postsynaptic density of neurons including protein kinase A, protein kinase C, mitogen-activated protein kinases, tyrosine kinases, phosphatases, PSD-95, and other PDZ domain containing proteins, small G proteins and their modulators, and the G protein-coupled metabotropic glutamate receptor (14). Evidence for large GPCR signaling complexes has been recently reviewed (2).

Here we have further explored the occurrence and nature of complex formation between GPCRs and Kir3 channels and adenyly cyclase. Catecholaminergic receptor signaling, particularly the β2AR and D2-dopamine receptors, have been used as prototypic models for GPCR signaling. Most commonly, signaling via the β2AR is described through its activation of the stimulatory G protein (Gsa) and adenyly cyclase, whereas of the D2 dopamine receptor is exemplified by its inhibitory action on the same effector via Gasi. However, both receptors are also recognized to be physiological regulators of the activity of G protein-activated inwardly rectifying potassium channels (GIRK or Kir3) (15–19). Here we show that both expressed GPCRs and effectors are part of stable complexes by demonstrating that Kir3 channels and adenyly cyclase co-precipitate with dopamine D2-like receptors and the β2AR. Furthermore, by using bioluminescence resonance energy transfer (BRET) we show that both adenyly cyclase and Kir3 channels are directly associated with the β2AR in living cells. The stability of these complexes is not altered by receptor activation or by inactivation of Go. However, whereas maintenance of the complex is not mediated by Gβγ, complex formation is in some cases Gβγ-dependent. These observations have important implications with regard to G protein-mediated signaling efficiency and specificity.

**EXPERIMENTAL PROCEDURES**

**Materials**

COS-7 cells were purchased from American Type Culture Collection (Rockville, MD). α-MEM was purchased from Central Media Preparation Service (University of Toronto, Toronto, ON). Fetal bovine serum, Dulbecco’s modified Eagle’s medium, LipofectAMINE reagent, and Opti-MEM I, geneticin (G418), T4 DNA ligase, T4 polynucleotide kinase, and oligonucleotides were bought from Invitrogen (Burlington, ON, Canada). All other DNA-modifying enzymes and restriction endonucleases were obtained from New England Biolabs (Beverly, MA). Isopropyl-β-D-thiogalactopyranoside was purchased from Molecular Probes (Eugene, OR). Low range molecular weight markers, nitrocellulose were from Bio-Rad. The protein solubilization reagent B-PER and BCA reagent for protein measurement were obtained from Pierce. Protein G-Sepharose, anti-His tag mouse monoclonal antibody, and glutathione-Sepharose 4B beads were from Amersham Biosciences. In some cases, pre-cast 10 and 8–10% gradient Tris glycine polyacrylamide gels were used, and these polyvinylidine difluoride membranes were purchased from Invitrogen. All other chemicals for SDS-PAGE were purchased from Bio-Rad. Dopamine, quinpirole, pertussis toxin (PTX), glutathione, aprotinin, leupeptin, phenylmethylsulfonyl fluoride, benzamidine, and soybean trypsin inhibitor were purchased from Sigma. Anti-FLAG (M2) mouse monoclonal antibody was purchased from Sigma, and anti-FLAG rabbit polyclonal antibody was obtained from Santa Cruz (Santa Cruz, CA). Digitonin and rat monoclonal anti-hemagglutinin (HA) (3F10) antibodies were obtained from Roche Molecular Biochemicals. Mouse monoclonal anti-β2 (Y11), anti-Myr (A14; 9E10) antibodies, and rabbit polyclonal anti-Gi antibodies were acquired from Santa Cruz Biotechnology. Mouse monoclonal anti-HA11 (16B12) antibody was from Berkeley Antibody Co. (Berkeley, CA). Monoclonal mouse anti-Kir3.1 (NH2-terminal) was from Upstate Biotechnology (Lake Placid, NY), and anti-Kir3.1 (COOH-terminal), anti-Kir3.2, and anti-Kv1.5 were from Alomone Labs (Jerusalem, Israel). Chemiluminescent detection of peroxidase-conjugated secondary anti-mouse or anti-rabbit antibodies (1:2000; Sigma) was performed using ECL Plus (Amersham Biosciences) or Renaissance (PerkinElmer Life Sciences). Plasmid vectors for the production of glutathione S-transferase (GST) fusion proteins were from the pGEX-series of Amersham Biosciences. The mammalian expression vector pCDNA3 was from Invitrogen. All other chemicals used were of reagent grade or higher and were obtained from Sigma.

**DNA Constructs**

The vector pCmDNA3 containing the dopamine D2α or D2 receptor cDNA with an amino-terminal, cleavable signal sequence immediately followed by the HA and FLAG epitopes (HA-D2α, FLAG-D2) have been described by us previously (20, 21). Epitope tags were engineered into human Kir3.1, Kir3.2e, Kir3.3, and Kir3.4 (22) by using standard molecular approaches employing the polymerase chain reaction. Oligonucleotides were designed to delete the existing termination codon in the channels and to add the required carboxyl-terminal epitope tag sequence followed by a termination codon. In this fashion we engineered Kir3.1 and Kir3.3 with a carboxyl-terminal HA tag (5'-TACCCGTATAGCTCCGCAGACTACCGCCG-3'), Kir3.2e with a carboxyl-terminal Myc tag (5'-GACACACACACACTCCTCAAGAGAGATCTG-3'), and Kir3.4 with a carboxyl-terminal FLAG tag (5'-GACTACAGGAGCAGCTGAGACGATGACAAG-3'). These constructs are denoted as Kir3.1-HA, Kir3.3-HA, Kir3.2-Myc, and Kir3.4-FLAG, respectively. For expression studies the tagged channels were cloned into the expression vector (23) pCmDNA3. The dominant negative G protein-α subunits described by Gilchrist et al. (23) were created as oligonucleotide sequences and subcloned into the vector pCmDNA3. The GST fusion protein for βARKct and the βARKct-mingine fire were a kind gift from Dr. R. L. Jefkowitz and Dr. W. J. Koch (Duke University). pCmDNA3, pRL-CMV, pGFP10, and pEGFP were obtained from Invitrogen, Promega (Madison, WI), Bio-Signal Packard (Montréal, QC, Canada), and Clontech, respectively. pHD4-A2-GFP10 was made by insertion into pGFP+3 (BioSignal Packard) βARKct-AR-Luc and pGFP10 for proteins consisting of GFP10 (a GFP variant described below) and Renilla reniformis luciferase (RLuc) fused to the COOH terminus of the human β2-adrener- gic receptor, respectively. βARKct-AR-Luc was constructed as follows: the pGFP10 Age/BsrG fragment was subcloned into the Age/BsrG1 site of pGFP-N1-Hisg-AR-TFP (24). pCmDNA3-βARKct-EGFP was a gift from J. Benovic (Thomas Jefferson University) as described (25). A plasmid containing the cDNA for the α-subunit of the stimulatory G protein (Gαs) was obtained from the American Type Culture Collection (ATCC number 63315). Plasmids containing the cDNA for the G protein γ2 subunit (γ2) were subcloned into pCmDNA3. A plasmid containing the cDNA for the bovine G protein β1 subunit (β1g, a gift from Arieh Katz [California Institute of Technology]) was subcloned into pCmDNA3. A plasmid containing the cDNA for the protein β2 subunit (β2) were subcloned into pCmDNA3. A plasmid containing the cDNA for the bovine β1 subunit (β1g, a gift from Arieh Katz [California Institute of Technology]) was subcloned into pCmDNA3. A plasmid containing the cDNA for the protein β2 subunit (β2) were subcloned into pCmDNA3. A plasmid containing the cDNA for the protein β2 subunit (β2) was subcloned into pCmDNA3. A plasmid containing the cDNA for the protein β2 subunit (β2) were subcloned into pCmDNA3. A plasmid containing the cDNA for the bovine β1 subunit (β1g, a gift from Arieh Katz [California Institute of Technology]) was subcloned into pCmDNA3. A plasmid containing the cDNA for the protein β2 subunit (β2) were subcloned into pCmDNA3. A plasmid containing the cDNA for the bovine β1 subunit (β1g, a gift from Arieh Katz [California Institute of Technology]) was subcloned into pCmDNA3. A plasmid containing the cDNA for the protein β2 subunit (β2) were subcloned into pCmDNA3. A plasmid containing the cDNA for the bovine β1 subunit (β1g, a gift from Arieh Katz [California Institute of Technology]) was subcloned into pCmDNA3. A plasmid containing the cDNA for the protein β2 subunit (β2) were subcloned into pCmDNA3. A plasmid containing the cDNA for the bovine β1 subunit (β1g, a gift from Arieh Katz [California Institute of Technology]) was subcloned into pCmDNA3. A plasmid containing the cDNA for the protein β2 subunit (β2) were subcloned into pCmDNA3. A plasmid containing the cDNA for the bovine β1 subunit (β1g, a gift from Arieh Katz [California Institute of Technology]) was subcloned into pCmDNA3.
by the supplier of the transfection reagent (Invitrogen). Transfection efficiency determined by using pCDNA3 expressing lacZ was about 60–80%. The cells were assayed or harvested for further experiments 2 days after transfection. For transient transfection of HEK 293 cells were seeded at a density of 10^5 cells/cm² in polycarbonate-coated plates and transiently transfected 24 h later with one or more plasmids using LipofectAMINE Plus (Invitrogen). Within experiments the concentration of DNA was kept constant by adding vector pCDNA3.1 or pCDNA3.1-luciferase. Sham transfections were also performed with these vectors.

**Membrane Solubilization and Immunoprecipitation**

D₁ and D₅ Dopamine Receptor Expressing COS-7 Cells—Prior to harvesting, cells were washed three times with ice-cold phosphate-buffered saline (PBS; pH 7.2) and subsequently collected by scraping in 10 ml of ice-cold PBS, spun at 1,200 rpm (5 min), and stored at −80 °C. The pellets were quickly thawed at room temperature and resuspended in 10 ml of TE buffer (10 mM Tris, pH 7.4, 10 mM EDTA, pH 8.0, 5 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μM phenylmethylsulfonyl fluoride). Next, the cells were homogenized on ice using a Polytron (2 × 5 s, maximum speed). The homogenate was spun for 3 min at 2,000 × g. The resulting supernatant was spun for 20 min at 34,000 × g. The pellet was solubilized in RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 1 mM EDTA, pH 8.0, 1% Nonidet P-40, 0.5% sodium deoxycholate, 5 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μM phenylmethylsulfonyl fluoride), and incubated at 4 °C for 1 h with gentle rocking. The resulting homogenate was spun for 10 min at 14,000 × g and the supernatant stored at −10 °C until assayed. For immunoprecipitation assays we used about 300 μg of total protein from the solubilized protein preparation as determined using the BCA protein assay (Pierce). The solubilized protein (300 μg) was aliquoted into microcentrifuge tubes and adjusted to a volume of 500 μl using RIPA buffer. Next we added 1 μg of the primary antibody to the samples and incubated the samples for 4 h at 4 °C with gentle rocking. To precipitate the antibody complexes in the mixture we added 40 μl of 50% Protein G-agarose slurry in RIPA buffer. This mixture was incubated for 3 h at 4 °C with gentle rocking. The beads (20 μl) were washed three times in RIPA buffer, incubated up to 50 μl of SDS loading buffer and stored at −20 °C until electrophoresis.

For dopamine D₁ receptor-Kir3.2 co-immunoprecipitation experiments from brain, rat striatal tissue (500 mg) was homogenized in buffer containing 50 mM Tris-Cl (pH 7.6), 150 mM NaCl, 1% Igepal CA630, 1% Triton X-100, 0.5% sodium deoxycholate, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and proteinase inhibitor mixture (Sigma, 5 μg/100 mg of tissue) for 1 h on ice. Next, the solubilized tissue was spun at 12,000 × g for 20 min. The supernatant was collected and protein concentrations were determined. For co-immunoprecipitation experiments, striatal lysates (500–700 μg of protein) were incubated with 1 μg of mouse anti-HIS tag, 1:400; anti-Kir3.1 (N), 1:200; anti-Kir3.1(C), 1:1000; or as negative control nonspecific normal rabbit serum (2 μg, Sigma, R91333) for 4 h at 4 °C, followed by the addition of 20 μl of protein A/G-Sepharose (Sigma) for 12 h. Pellets were washed four times in buffer as described above,boiled for 5 min in SDS sample buffer, and subjected to SDS-PAGE. The D₁ receptor was detected on Western blots with an anti-D₁ antibody as recommended by the manufacturer (D2R14-A, BioTrend Chemikalien, Germany).

**BRET Analysis**

BRET was performed as described previously (24) with the following modifications. D4.2-FFP10, β₂AR-GFP, or β₂AR-YFP fusion proteins were co-expressed with the luciferase reporter in HEK 293 cells. Cells were transfected with 6-well culture plates and transiently transfected with the relevant constructs. Forty-eight hours post-transfection, HEK-293T cells were washed twice in PBS, detached with PBS, and resuspended in PBS containing 0.1% bovine serum albumin and 0.01% fatty acid-free gelatin. Cells were incubated in 5% carbon dioxide for 1 h at 37 °C and washed three times in serum-free conditions. Prior to electrophoresis the protein samples taken up in SDS-loading buffer were heated for 2–5 min at 80 °C and spun quickly at 14,000 × g. Samples were size separated by gel electrophoresis using 10–16% gradient Tris glycylicrylamide gels, followed by transfer of the membrane to nitrocellulose or polyvinylidene difluoride membranes. For immunological detection of material, nonspecific binding to polyvinylidene difluoride or nitrocellulose membranes was blocked by overnight pretreatment of the membrane with blocking buffer containing 5% nonfat dry milk powder in Tris- or phosphate-buffered saline with 0.2% Tween 20 (TBS-T or PBS-T). The membranes were incubated for different times (dependent on the primary antibody used) with the appropriate dilution of primary antibody in blocking buffer (anti-HA (3F10), 1:2000; anti-HA.11 (16B12), 1:1000; anti-e-Myc (A14), 1:400–1000; anti-c-Myc (9E10) 1:200; anti-FLAG (M1 and M2) 10 μg/ml; anti-HIS tag (Novagen, 1:2000); anti-Ki3.1 (N), 1:200; anti-Ki3.1(C), 1:1000; anti-Ki3.2, 1:1000). After incubation with the primary antibody the membranes were washed extensively with TBS-T or PBS-T, and secondary antibody detection was performed using standard protocols with peroxidase-conjugated anti-IgG and enhanced chemiluminescence.
effective quantification of protein–protein interactions. Similar results were obtained when using BRET3 (EGFP or YFP) or BRET4 (GFP10). The BRET signal generated is calculated by the ratio of light emitted by the GFP partner over the light emitted by the RLuc partner. Deep Blue C coelenterazine (for BRET3; BioSignal Packard Bioscience) or coelenterazine H (for BRET4; Molecular Probes) were added at a final concentration of 5 μM. Signals were collected on a Packard Fusion instrument using either 410/80-nm (luciferase) and 515/30-nm (GFP) band pass filters for pβ2AR-GFP10 or 470/60 (luciferase) and 550/60-nm band pass filters (GFP) for pβ2AR-YFP. Whether or not BRET occurred was determined by calculating the ratio of the light passed by the 515/30 filter to that passed by the 410/80 or 550/80 to the 470/60 filter. This ratio is referred to as the BRET ratio.

Receptor Modulation

Agonist treatment of dopamine receptor expressing cells was done with 1 μM dopamine or quinpirole for 5 min at 37 °C, 2 days after transfection. For PTX treatment the cells were washed with serum-free α-MEM 1 day after transfection, and subsequently the cells were grown for another day in α-MEM containing 100 ng/ml PTX. After treatment, the plates on which the cells were grown were washed three times with ice-cold PBS and cells were harvested by scraping and stored at −20 °C. To block trans-Golgi transport of newly synthesized material 5 h after transfection, growth media was supplemented with 10 μg/ml brefendin A. After incubation for 16–20 h with brefendin A cells were harvested as described above. In the case of β2AR expressing cells, 10 μM isoproterenol was also included in all solutions used for cell harvesting and solubilization.

For the β2AR-ct-mediated Gfy competition experiment we purified GST and GST-β2ARct from Escherichia coli strain BL21-Codon-Plus-RIL containing pGEX3 plasmid constructs expressing the GST fusion proteins. The proteins were isolated using B-PER (Pierce) and glutathione-Sepharose 4B beads as recommended by the manufacturer. A solubilized membrane preparation expressing HA-tagged D2 receptors and c-Myc-tagged Kir3.2c were incubated with 0.2 μM of the GST fusion proteins during the immunoprecipitation protocol.

Receptor Binding and cAMP Assays

Cells grown in 24-well tissue culture plates were assayed as previously described (27) to determine the number of β2-adrenergic receptors. Binding of the hydrophilic ligand (−)-[125I]cyanopindolol (CYP) and (−)-[3H]CGP12177 (CGP) were used to determine the total number and cell surface receptors, respectively. Nonspecific binding was determined in the absence or presence of 10 μM (-)-propranolol. Cells were also assayed for basal and agonist-stimulated levels of cAMP. To block trans-Golgi transport of newly synthesized material 5 h after transfection, growth media was supplemented with 100 ng/ml PTX. After treatment, the plates on which the cells were grown were washed three times with α-MEM containing 100 ng/ml PTX. After treatment, the plates on which the cells were grown were washed three times with ice-cold PBS and cells were harvested by scraping and stored at −20 °C. To block trans-Golgi transport of newly synthesized material 5 h after transfection, growth media was supplemented with 10 μg/ml brefendin A. After incubation for 16–20 h with brefendin A cells were harvested as described above. In the case of β2AR expressing cells, 10 μM isoproterenol was also included in all solutions used for cell harvesting and solubilization.

Expression in Xenopus Oocytes and Electrophysiological Recording

Kir3 channel cDNAs were linearized and cRNA were synthesized and expressed in Xenopus oocytes as described previously (16, 22). cDNA constructs for various tagged Kir3 (Kir3.1, Kir3.2c, and Kir3.4) channel isoforms and the HA-D2, D2-GFP10, β2AR-EGFP, and β2AR-GFP10 constructs were linearized by restriction enzymes and purified using GeneClean (Bio 101, Vista, CA; Capped mRNA was made using T7 RNA polymerase and the mMessage mMachine (Ambion). Individual oocytes were injected with 5–10 ng (in 25–50 nl) of each cRNA. Recordings were made 48 h after injection at room temperature using a Geneclamp 500 amplifier (Axon Instruments). Oocytes were voltage clamped and perfused continuously with different recording solutions. Data was recorded at a holding potential of −80 mV or from +80 mV to −140 mV in 20-mV steps. Drugs were added to the bath with a fast perfusion system. Data collection and analysis were performed using pCLAMP v6.0 (Axon Instruments) and Origin v4.0 (MicroCal) software.

RESULTS

Expression and Function of Tagged GPCRs and Effectors—

Previous studies have shown that covalently coupling either a GFP variant or RLuc to the COOH terminus of the β2AR does not affect its affinity for ligands or the efficacy with which the receptor mediates hormonal stimulation of AC (24, 25, 28). The expression of β2AR-EGFP resulted in fluorescence that was associated with internal membranes and with the plasma membrane (data not shown). Ligand binding experiments revealed that all of the detectable endogenous β2-adrenergic receptors in HEK 293 cells are present on the cell surface. Transfected HEK 293 cells expressed 2.8 ± 0.1 pmol of β2AR-EGFP per mg of cell protein, and 43 ± 7% of the expressed receptors were on the cell surface. Thus, the distribution of binding sites reflected the distribution of fluorescence, and indicated that the β2AR-EGFP, associated with both the plasma membrane and internal membranes, was capable of binding ligand. Similar results were obtained for β2AR-YFP (24) and β2AR-GFP10 (data not shown), which were used in BRET assays.

Epitope tagging the NH2 terminus of D4L and D3 receptors did not affect their function as determined by receptor-mediated regulation of downstream effectors (Ref. 21, and data not shown). BRET experiments require that the proteins being studied have either GFP or RLuc fused to them. Because fusing different GFP variants (EGFP, YFP, and GFP10) to the COOH terminus of the β2AR did not oblate its function, a similar approach was taken in making a fusion protein of dopaminergic receptor and GFP. However, this rendered the dopaminergic receptor inactive as determined by its inability to activate G proteins or regulate effectors. It seemed unlikely that fusing RLuc to the COOH terminus would result in a functional dopaminergic receptor, and no further attempts were made to intercalate GFP or RLuc into the receptor at a point where it would not disrupt receptor function.

Based on biochemical and structural information, it was decided that attaching RLuc to the COOH terminus of AC would be least likely to disrupt its function. Hormone-induced accumulation of cAMP by cells expressing β2AR-EGFP either with or without GαS (i.e. GαS(βγ)) was increased more than 6-fold by co-expression of AC-RLuc indicating that the AC-RLuc fusion protein retained its ability to function in G protein-mediated signal transduction (Table I).

To simplify biochemical analysis, four different human Kir3 channel subunits were altered by the addition of epitope tags to their COOH-terminal ends (Kir3.1-HA, Kir3.2-Myc, Kir3.3-HA, and Kir3.4-FLAG). When expressed in COS-7 cells the individual channel subunits were readily detectable by Western analysis with antibodies against the different epitope tags (Fig. 1A). The migration pattern of the epitope-tagged channels corresponds closely to the molecular mass predicted from the cloned channels (Kir3.1, 56.1 kDa; Kir3.2c, 48.5 kDa; Kir3.3, 44 kDa; Kir3.4, 47.5 kDa). The migration of Kir3.1 at slightly higher than predicted molecular weight may be because of its reported glycosylation. We and others have found that the addition of epitope tags to Kir3 channel subunits do not affect their ability to assemble into functional channels (Ref. 29, and
Expression and co-immunoprecipitation of human Kir3 channel subunits in COS-7 cells. A, COS-7 cells transfected with individual epitope-tagged Kir3 channel subunits were readily detected by Western analysis using antibodies directed to the Kir COOH-terminal fused epitope tags HA, FLAG, and c-Myc (Kir3.1-HA, Kir3.2-Myc, Kir3.3-HA, and Kir3.4-FLAG). * indicates the detection of endogenously expressed c-Myc. B, COS-7 cells were transfected with two different Kir3 channel subunits and tested for co-immunoprecipitation. All Kir3 family members co-immunoprecipitated with each other (only a few examples are shown), no matter which partner is immunoprecipitated (not shown). Kir channels that were expressed separately and mixed during protein extraction (†) showed no co-immunoprecipitation. There was no cross-reactivity between the different antibodies and the epitopes employed in this study (not shown). The figure is representative of at least three separate experiments. The different antisera used for immunoprecipitation (IP) and immunoblotting (IB) are indicated.

A recombinant expression vector encoding a fusion protein composed of the Kir3.1 channel subunit and RLuc was prepared. In experiments performed with Xenopus oocytes, co-expression of Kir3.1-RLuc with Kir3.4 resulted in the formation of functional inwardly rectifying potassium channels that can be activated by β2AR-EGFP, and were essentially indistinguishable from wild-type Kir3.1/Kir3.4 channels under two-electrode voltage clamp (data not shown). Previous studies have also demonstrated that COOH-terminal fusions of GFP to Kir3.1 did not affect channel function (30). These experiments, which demonstrated that the various receptors, Kir3, and adenylyl cyclase fusion proteins are functional, allowed us to proceed with studies of the physical interactions between these receptors and their effectors.

Co-immunoprecipitation of GPCRs with Their Cognate Effectors—Immunoprecipitation of epitope-tagged dopaminergic receptors or β2AR-GFP fusion proteins with the appropriate antibody resulted in the precipitation of co-expressing Kir3 channel subunits (Figs. 2, 3, and 4). Reversing the approach and immunoprecipitating the Kir3 channel subunits resulted in precipitation of the co-expressed receptors (Fig. 2, A and D). The D2 receptor migrates in the Western analysis close to its predicted molecular weight of 44,000. As expected, the β2AR-GFP fusion protein runs at a higher molecular weight (Mw) than the β2AR (predicted Mw, 46,000), however, it migrates at a lower Mw than predicted for the fusion protein (His-β2AR-GFP, 76,500). Whether this constitutes a gel running artifact because of structural features of the fusion protein is unknown. Considering that both the His tag and GFP portion are still functional for the modified β2AR it is unlikely because of proteolytic digestion. The specificity of the receptor/channel interaction is highlighted by the observation that separate expression of the receptors and the Kir3 channels and subsequent solubilization, mixing of membrane preparations, and receptor immunoprecipitation did not result in co-association of Kir3 (Fig. 2A). Furthermore, when co-expressed, FLAG-D2 receptors did not immunoprecipitate a Myc epitope-tagged version of the closely related inwardly rectifying potassium channel Kir2.1 (Kir2.1-Myc), suggesting that these receptors do not indiscriminately associate with all Kir channels family members (Fig. 2C).

Experiments were also done to determine whether immunoprecipitation of β2AR-GFP would result in the co-precipitation of AC-RLuc. The membranes of cells expressing β2AR-GFP together with either soluble RLuc or AC-RLuc were dissolved in various detergent-containing solutions and immunoprecipitates were prepared using antibodies against GFP. RLuc was co-precipitated with β2AR-GFP from cells co-expressing AC-RLuc but not from cells co-expressing RLuc if the cell membranes were dissolved in a solution containing 1% digitonin (Table II). When other detergents (i.e. n-dodecyl-β-D-maltoside, Lubrol PX, sodium cholate, or a mixture of Triton X-100 and sodium cholate) were used to dissolve cell membranes no co-precipitation of AC-RLuc with the β2AR-GFP was observed. These results are consistent with previous studies showing that preservation of lipophilic protein complexes is largely dependent on the type of detergent used to dissolve the membranes.

Experiments were then performed to verify that these interactions also occur in native tissue. Dopamine D2 receptors could be co-immunoprecipitated from brain (striatum) with Kir3.2 (Fig. 3A, right panel). In negative control experiments, using a nonspecific IgG antiserum, D2 receptors were not immunoprecipitated. The specificity of the anti-D2 antibody is shown by using Western analysis of HEK 293 cells transfected with HA-tagged D2 receptors or vector as negative control (Fig. 3A, left panels). β2AR were immunoprecipitated from mouse heart or brain, and samples were blotted for β2AR (to verify immunoprecipitation, Fig. 3B, left panel), adenylyl cyclase VVI (expressed ubiquitously), and Kir3.2. In both heart and brain extracts, the β2AR could co-immunoprecipitate adenylyl cyclase (Fig. 3B, middle panel). In brain tissue, Kir3.2 was also co-immunoprecipitated with β2AR (Fig. 3B, right panel). As a negative control, we attempted to co-immunoprecipitate Kv1.5, another membrane protein highly expressed in both tissues. The anti-β2AR antibodies could not immunoprecipitate.
Kv1.5 from either tissue (data not shown) demonstrating the specificity of the interaction in vivo. In the Western analysis the migration of the native receptors and heterologously expressed D2 receptors is at slightly higher than predicted molecular weights, which is in concordance with the reported glycosylation of these proteins.

Neither Agonist Treatment nor ADP-ribosylation of G\textsubscript{i} by PTX Affects Co-precipitation of GPCRs with Their Effectors—To determine whether agonist-mediated activation of signal transduction affects co-precipitation of receptors and effectors, cells expressing Kir3.2-Myc channel subunits and either epitope-tagged dopamine D\textsubscript{2L} or D\textsubscript{4} receptors were incubated with 1 \muM dopamine or quinpirole. Similarly, we co-expressed epitope-tagged \beta\_AR with Kir3.1 or Kir3.2c and explored whether stimulation with 10 \muM isoproterenol would affect receptor-channel co-immunoprecipitation. Treatment with either agonist for 5 min is sufficient for maximal receptor-mediated activation of its effectors. The receptors were subsequently immunoprecipitated from agonist-treated cells and the amount of co-precipitated Kir3.2c was compared with that obtained from unstimulated cells. Coprecipitation of Kir3.2 channel subunits with epitope-tagged D\textsubscript{4} receptors (Fig. 4) or \beta\_AR (Fig. 5) was largely unaffected by receptor agonists, although subtle changes cannot be excluded from these experiments. The GFP fusion constructs of \beta\_AR that were not His-tagged served as negative controls showing the specificity of the protocol used to demonstrate co-immunoprecipitation of Kir3.1 with His-tagged \beta\_AR-GFP (Fig. 5).

**Fig. 2.** Co-immunoprecipitation of catecholamine receptors with Kir3 channel subunits. Cells (COS-7 for D\textsubscript{2} and D\textsubscript{4} receptors; HEK 293 for \beta\_adrenergic receptors) were co-transfected with either NH\textsubscript{2}-terminal FLAG- or HA epitope-tagged D\textsubscript{2} receptors (FLAG-D\textsubscript{2}, HA-D\textsubscript{2}) (A and C), NH\textsubscript{2}-terminal HA epitope-tagged D\textsubscript{4} (HA-D\textsubscript{4}) (B) or NH\textsubscript{2}-terminal His- and COOH-terminal GFP-tagged \beta\_adrenergic receptors (His-\beta\_AR-GFP) (D) as well as with wild-type Kir3.1, COOH-terminal HA epitope-tagged Kir3.1 (HA-D\textsubscript{2}) and Kir3.3-HA, Myc epitope-tagged Kir3.2c (Kir3.3-Myc), FLAG epitope-tagged Kir3.4 (Kir3.4-FLAG), and Myc epitope-tagged Kir2.1 (Kir2.1-Myc) channel subunits. Co-immunoprecipitation of the different Kir subunits (as indicated by the arrows in the blots) with antisera directed to the tagged receptors is shown. Different cell populations were transfected separately with FLAG-D\textsubscript{4} and Kir3.2-Myc and mixed just prior to solubilization (lane indicated with †; FLAG-D\textsubscript{4} and Kir3.2-Myc). In a reverse immunoprecipitation protocol the FLAG-D\textsubscript{4} could be co-immunoprecipitated with Kir3.2-Myc (A). HA-D\textsubscript{2} receptors co-immunoprecipitate the different Kir3 channel subunits (B). Co-expressed HA-D\textsubscript{2} and Kir2.1-Myc channel subunits could not be co-immunoprecipitated (C, last two lanes), despite equivalent high expression of both proteins as detected in whole cell lysates (C, first two lanes). His-\beta\_AR-GFP could co-immunoprecipitate Kir3.2 with FLAG-D\textsubscript{4} compared with cells co-transfected with both constructs (FLAG-D\textsubscript{4} + Kir3.3-Myc). In a reverse immunoprecipitation protocol the FLAG-D\textsubscript{4} could also be co-immunoprecipitated with Kir3.3-Myc (A). HA-D\textsubscript{2} receptors co-immunoprecipitate the different Kir3 channel subunits (B). Co-expressed HA-D\textsubscript{2} and Kir2.1-Myc channel subunits could not be co-immunoprecipitated (C, last two lanes), despite equivalent high expression of both proteins as detected in whole cell lysates (C, first two lanes). His-\beta\_AR-GFP could co-immunoprecipitate Kir3.2 with FLAG-D\textsubscript{4} (D). In a triple co-transfection protocol in which His-\beta\_AR-GFP, Kir3.1, and Kir3.4-FLAG were co-expressed we could effectively co-immunoprecipitate Kir3.4-FLAG with His-\beta\_AR-GFP (D). In reverse co-immunoprecipitation protocols His-\beta\_AR-GFP could also be immunoprecipitated with the Kir3.4-FLAG and Kir3.1-HA channel subunits, whereas in cells transfected with untagged versions of these channels the receptor could not be immunoprecipitated (D). The figure is representative of at least three separate experiments. Tests using nonspecific antibodies as controls were negative for co-immunoprecipitation (not shown). The different antisera used for immunoprecipitation (IP) and immunoblotting (IB) are indicated.
Because the activation of dopamine D₂, and D₄ receptors and consequent opening of Kir3.2c channels is sensitive to ADP-ribosylation of Gαᵪ by PTX, we analyzed whether treatment with this toxin altered receptor/channel interactions. Although the conditions employed in this study block dopamine D₂, and D₄ receptor mediated-signal transduction (Ref. 21, and data not shown), it did not alter the amount of dopamine D₂, and D₄ receptor that was co-precipitated with Kir3.2c channels compared with nontreated cells (Fig. 4). The lack of PTX effect on co-precipitation of Kir3.2c with dopamine receptors indicates that a functional Gαᵪ is not required for the interaction between dopaminergic receptors and Kir3 channel subunits. This observation is also consistent with the lack of an effect of agonist treatment on receptor-channel co-immunoprecipitation. This is further supported by the observation that co-expression of dominant-negative Gαᵪ constructs (23), which can block GPCR-mediated activation of Kir3 channels, did not markedly change the dopamine receptor-Kir3.2c complex formation either (Fig. 6A). These dominant-negative Gαᵪ constructs consist of a short carboxyl-terminal fragment of the G protein that competes with the G protein for interactions with the receptor and thus blocks receptor-mediated activation of Kir3 channels.

Evidence for Assembly of GPCR-Effector Complexes Prior to Their Incorporation into the Plasma Membrane and a Potential Role for Gβγ in the Assembly Process—It has been well described that the activation of Kir3 channels is Gβγ-dependent (31). It is also known that expression of the COOH-terminal domain of G protein-coupled receptor kinase (βARKct) binds Gβγ and interferes with channel activation (32). However, it is not known whether the βARKct can interfere with formation of the receptor-channel complex. Co-expression of βARKct with Kir3.2c channels and D₂ receptors results in a dramatic loss of receptor-channel complex formation (Fig. 6A). Considering that modulation and disruption of GPCR signaling by PTX and agonist treatment did not affect markedly the co-immunoprecipitation of the receptor-channel complex we postulated that Gβγ plays an early role in the complex formation. To determine whether the receptor-channel complex is formed during biosynthesis (rather than at the plasma membrane) we incubated cells with brefeldin A shortly after transfection to block transport of newly synthesized proteins from Golgi to trans-Golgi. This treatment did not block complex formation as evidenced by co-precipitation of epoepitope-tagged Kir3.2c channel subunits with the epitope-tagged D₂, and D₄ receptor, indicating that the receptor-channel complex forms prior to its appearance at the cell surface (Fig. 6A). Furthermore, brefeldin A treatment did not prevent the βARKct-mediated block of the receptor-channel complex formation (Fig. 6A), suggesting that the Gβγ subunit plays a role in early complex formation (Fig. 6A). To determine whether the Gβγ subunits are critical for the maintenance of the dopamine receptor-Kir3.2c complex we added an excess of purified GST-βARKct to the solubilized membrane preparation. The addition of the βARKct did not cause the dissociation

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of the dopamine receptor-Kir3.2c complex (Fig. 6B), suggesting that the βARKct cannot destabilize the receptor-effector complex once it is formed. Of relevance in this regard is our observation that D₄ receptors and Kir3 channels co-precipitated under the conditions used for our experiments were not associated with detectable amounts of Gβγ (Fig. 6B). Taken together these data strongly support the view that Gβγ plays a role in early complex formation rather than maintenance of the complex.

**BRET Provides Evidence for Direct Interaction of GPCRs with Their Cognate Effectors in Vivo That Survives Agonist Occupancy of the Receptor**—BRET occurs if the distance between donor (RLuc) and acceptor (GFP) is less than 100 Å. By and large, the distance between two individual proteins is less that 100 Å only if they are directly associated with each other. Furthermore, BRET can be assayed in living cells making it useful for determining if stable protein/protein interactions occur in vivo. Being able to attach the GFP or RLuc to receptors and effectors without disrupting their ability to engage in G protein-mediated signal transduction paved the way for the use of BRET to determine whether GPCRs and their cognate effectors form stable signaling complexes in vivo.

We have previously demonstrated that human Kir3.1 requires co-expression of the Kir3.2c or Kir3.4 subunits to become a functional channel (22). BRET was observed between Kir3.1-RLuc and the β₂AR-GFP10 when cells co-expressed either Kir3.2c or Kir3.4 (Fig. 7A) and there were no changes in the BRET ratio in response to agonist, again suggesting that receptor and effector are associated with one another regardless of whether or not signal transduction is activated (Fig. 7B). In the absence of co-expressed Kir3.2c or Kir3.4 BRET between Kir3.1-RLuc and the β₂AR-GFP10 is markedly reduced, suggesting that the presence of a functional channel improves or stabilizes the interaction with the receptor (Fig. 7A). BRET also occurred when β₂AR-GFP10 and AC-RLuc were co-expressed regardless of whether or not the cells were treated with agonist (Fig. 7, A and B), suggesting that the receptor and effector interact directly with one another in living cells, and that the interaction is not dependent upon activation of the signal transduction pathway. It has been demonstrated that the β₂AR forms oligomers in vivo by showing that BRET occurs when cells co-express β₂AR-YFP and β₂AR-RLuc (24). We used co-expression of β₂AR-GFP10 and β₂AR-RLuc as a positive control for our experiments and observed BRET as expected (Fig. 7A). Finally, there was no BRET when the functionally inactive D₄-GFP10 fusion protein was co-expressed with Kir3.1-RLuc and Kir3.4, a subunit combination that produces functional heterotetrameric Kir3 channels (data not shown). The BRET ratio was the same for cells co-expressing soluble GFP10 and effector-RLuc fusion proteins or β₂AR-GFP10 and soluble RLuc, as it was for cells expressing RLuc alone or RLuc fusion proteins alone, indicating the specificity of the assay and that BRET did not occur between donor and acceptor if the proteins did not associate.

**DISCUSSION**

Co-immunoprecipitation experiments were used to demonstrate that three different GPCRs (D₂L and D₄ dopamine receptors and the β₂AR) stably interact with their downstream effectors including several inwardly rectifying potassium channel (Kir3) subunits and adenylyl cyclase. Co-precipitation of effectors with their receptors occurred regardless of whether or not signal transduction was activated. Co-precipitation of effectors with receptors suggests that these complexes also exist in living cells, which was confirmed using BRET. As with the immunoprecipitable complexes, the interaction of receptors and effectors in living cells was stable in the absence as well as the presence of agonists. With the exception of D₄-GFP, all of our fusion proteins were functional and had properties similar to their untagged counterparts. Expressing Kir3 channels and dopaminergic receptors in separate cells and mixing the detergent-soluble material from these cells did not result in the formation of complexes containing receptors and effectors, suggesting that the assembly of these complexes is a biosynthetic event. This observation is further supported by evidence that the trans-Golgi transport blocker brefeldin A is ineffective in preventing receptor-channel complex formation, which supports the view that the association of a receptor with its effector occurs prior to their appearance at the cell surface. Receptor-effector complexes were also detected in native tis-
This indicates that the receptor/effector interactions are of physiological relevance and not merely a consequence of the conditions of the heterologous expression systems. Specifically, we have provided evidence for the existence of dopamine D2 receptor-Kir3.2 complexes in mouse brain (striatum), and β2AR-Kir3.2 and adenylyl cyclase V/VI complexes in mouse heart and brain tissues.

The observation that the receptors can co-immunoprecipitate with nonfunctional homomeric Kir3.1 or Kir3.3 channels suggests that functionality of the receptor-channel complex may not be a prerequisite for assembly of this complex. This is also supported by data in which D4 mutants unable to promote G protein GDP-GTP exchange can co-immunoprecipitate Kir3.2 (data not shown). Furthermore, homomeric Kir3.1, which forms a nonfunctional channel, can mediate a modest BRET signal when expressed with β2AR. In addition, a functional transducer did not appear to be necessary for complex formation because neither PTX treatment nor expression of dominant negative Gαi constructs prevented co-precipitation of dopaminergic receptors and Kir3 channels. However, the experiments do not exclude subtle changes in the efficacy of the interaction. Similarly, Gβγ does not appear to be necessary for maintenance of the mature signaling complex. Note, however, that whereas we were unable to implicate Gβγ in the stability of a receptor-effector complex once it was formed, we did find evidence of a role for the G protein heterodimer in facilitating assembly because neither PTX treatment nor expression of dominant negative Gαi1/2 nor expression of dominant negative Gβγ sequestering protein (βARKct) results in a complete loss of receptor-channel complex formation. The absence or presence (+ or −) of brefeldin A (10 μg/ml, overnight) was without effect on the co-immunoprecipitation. Preincubation of GST-βARKct during protein extraction of transfected COS-7 cells with D4 receptors and Kir3.2c channel subunits causes no dissociation of the complex. Cells were co-transfected with FLAG-tagged D4 and Myc-tagged Kir3.2c (Kir3.2-Myc) expression vectors. Two days after transfection cells were lysed in the presence of either GST or GST-βARKct for 1 h at 4 °C. Control GST-βARKct, unlike GST, shows the association with Gβγ. No difference in the levels of co-immunoprecipitation of the receptor-channel complex was observed when GST was included in the co-immunoprecipitation protocol. The figure is representative of at least three separate experiments. The different antibodies used for co-immunoprecipitation (IP) and immunoblotting (IB) are indicated.

**Fig. 6.** The role of heterotrimeric G protein subunits in dopamine receptor/Kir3 association. A, the effect on co-immunoprecipitation of the dopamine receptor-Kir3 complex by co-expression of dominant negative Gαi protein (D.N. Ga-i1/2 and D.N. Ga-i3), and Gβγ sequestering protein (βARKct) was measured with NH2-terminal FLAG-tagged dopamine D4 receptors (FLAG-D4) and COOH-terminal Myc-tagged Kir3.2c (Kir3.2-Myc) channels in COS-7 cells. For expression, the dominant-negative expression and control constructs (pCDNA3) were transfected at a 10-fold higher molar ratio than the receptor and channel expressing constructs. Overexpression of βARKct results in a complete loss of receptor-channel complex formation. This indicates that the receptor/effector interactions are of physiological relevance and not merely a consequence of the conditions of the heterologous expression systems. Specifically, we have provided evidence for the existence of dopamine D4 receptors and Kir3.2c channel subunits causes no dissociation of the complex. The presence or absence (+ or −) of brefeldin A (10 mg/ml, overnight) was without effect on the co-immunoprecipitation. B, preincubation of GST-βARKct during protein extraction of transfected COS-7 cells with D4 receptors and Kir3.2c channel subunits causes no dissociation of the complex. Cells were co-transfected with FLAG-tagged D4 and Myc-tagged Kir3.2c expression vectors. Two days after transfection cells were lysed in the presence of either GST or GST-βARKct for 1 h at 4 °C. Control GST-βARKct, unlike GST, shows the association with Gβγ. No difference in the levels of co-immunoprecipitation of the receptor-channel complex was observed when GST was included in the co-immunoprecipitation protocol. The figure is representative of at least three separate experiments. The different antibodies used for co-immunoprecipitation (IP) and immunoblotting (IB) are indicated.
manipulations that affect the functional activity of the mature complex. Nevertheless, some of the data support that functionality may be a contributing factor with regards to the efficacy of complex formation. This is revealed by the observations that D_{2}GFP10, which does not functionally couple to its effectors, does not mediate BRET with Kir3.1-Kir3.4 channel complexes. Furthermore, the co-expression of Kir3.1 with Kir3.4 or Kir3.2, rather than Kir3.1 by itself, significantly enhanced the BRET signal with β_{2}AR. Similarly, Kir2.1 channels, which are not direct effectors of dopamine receptors, did not form complexes with D_{2} receptors. Whether these differences in receptor-channel complex formation are related to a more efficacious assembly of the complex or merely represent improved expression because of either increased stability or decreased degradation of the constituents of the complex is as of yet unknown.

The co-immunoprecipitation protocols for the receptor-channel complexes using “strong” detergents (RIPA) in combination with the BRET data support the view that the receptor-channel complex is mediated by direct protein/protein interactions. However, it cannot be excluded that the co-immunoprecipitation and BRET of the receptor-effector complexes is a result of the co-localization of the signaling partners into lipid microdomains or rafts (33).

How Gβγ is involved in receptor-channel complex formation during early synthesis is of yet unknown. G proteins have been implicated in the maintenance of the integrity of the Golgi system and regulation of trafficking in this system (34, 35–37). Because the inhibitory effects of βARKct on complex formation were brefeldin-insensitive an indirect role of Gβγ via interference with the Golgi system seems unlikely, supporting a more direct role in complex formation. However, it has also been reported that Gβγ heterodimers are assembled in the cytosol and that their final targeting to the plasma membrane is not affected by brefeldin (38). These different observations are difficult at present to reconcile with a mechanism on how Gβγ plays a role in receptor-channel complex formation.

The existence of stable receptor/G protein interactions as well as G protein-effector complexes have been reported by many investigators (see Ref. 2 for review). Our findings extend this paradigm to include direct interactions between receptors and their associated effector molecules. The concept of a signaling complex containing many of the components involved in G protein-mediated signal transduction is supported by data demonstrating that the βγ-AR can be co-precipitated from rat brain with one of its effectors, the voltage-gated L-type calcium channel Ca_{1.2} (8), and that the precipitate includes heterotrimeric G proteins, AC, protein kinase A, and phosphatase PP2A. Indeed, recent work has shown that molecular scaffolds such as protein kinase A-binding proteins in addition to scaffolding protein kinase A may also function as anchors for larger signaling complexes (8, 39, 40). However, our data also suggest that the G proteins may not be essential for maintenance of the complex.

Neurons or cardiomyocytes contain many different receptors, G proteins, and effectors that would have to transiently associate, dissociate, and then subsequently re-associate after each activation cycle. Such a scheme is difficult to reconcile with our current notions of signaling specificity in these cells. We have demonstrated that stable signaling complexes exist between three different GPCRs and their downstream effectors, suggesting that assembly of these signaling complexes is likely to be a biosynthetic event, rather than ones directed by agonist at the cell surface. The exact constituents of each complex may depend on the specific cell, cell type and/or tissue type studied. Preassembly of signaling components into a specific subcellular location and/or macromolecular complex may provide a means of insuring specificity and rapidity of response (41).

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