The G Protein α Subunit Has a Key Role in Determining the Specificity of Coupling to, but Not the Activation of, G Protein-gated Inwardly Rectifying K⁺ Channels*

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In neuronal and atrial tissue, G protein-gated inwardly rectifying K⁺ channels (Kir3.x family) are responsible for mediating inhibitory postsynaptic potentials and slowing the heart rate. They are activated by Gβγ dimers released in response to the stimulation of receptors coupled to inhibitory G proteins of the Gi/o family but not receptors coupled to the stimulatory G protein Gs. We have used biochemical, electrophysiological, and molecular biology techniques to examine this specificity of channel activation. In this study we have succeeded in reconstituting such specificity in an heterologous expression system stably expressing a cloned counterpart of the neuronal channel (Kir3.1 and Kir3.2A heteromultimers). The use of pertussis toxin-resistant G protein α subunits and chimeras between Gi1 and Gi indicate a central role for the G protein α subunits in determining receptor specificity of coupling to, but not activation of, G protein-gated inwardly rectifying K⁺ channels.

G protein-gated, inwardly rectifying K⁺ channels were first identified in atrial myocytes where they were shown to be activated by acetylcholine at muscarinic m₂ receptors (1), and stimulation of this current is responsible for slowing of the heart rate in response to vagal nerve stimulation. It was subsequently shown that this activation was sensitive to pertussis toxin (PTx), implicating the family of Gi/Go proteins (2–4). It is now apparent that G protein-gated inwardly rectifying K⁺ currents are present in many neuronal cell types, and they are involved in the postsynaptic inhibitory effects of stimulating Gi/Go-coupled receptors such as GABA_B and adenosine A₁ (5).

Cloning efforts from a number of laboratories have revealed the molecular counterparts of these currents (6–10). The channel is a heteromultimer of members of the inwardly rectifying Kir3.x family of potassium channels. Co-expression of Kir3.1 with Kir3.2, Kir3.3, or Kir3.4 results in currents that show many of the basic characteristics of the native channels in neurons and atria (11–13). Homomultimers of splice variants of Kir3.2 may also form in certain neuronal regions (14).

Channel activation of these currents in native tissues and of the cloned counterparts in heterologous expression systems was shown to be membrane-delimited (4, 15), involving a direct interaction with the G protein βγ dimer (16). This finding was initially controversial, but there are now numerous lines of evidence that point to the involvement of the βγ subunit as the direct channel activator (17). Recent studies have focused on domains and residues involved in G protein βγ dimer binding (18–24) and the cell biology of channel biogenesis (25–27).

The question remains as to how receptor specificity is achieved and what are the key determinants involved in this. In native tissues only Gi/o-coupled receptors, and not Gs-coupled receptors, activate these channels, and yet both should liberate free G protein βγ upon receptor stimulation. Receptor selectivity does not lie at the level of the G protein βγ dimer since a variety of different βγ subunit combinations have been shown to be similarly effective at potentiating these currents (28). In this study we have used a mammalian expression system (HEK293 cells) to investigate the neuronal-type Kir3.1+3.2A channels and their modulation by a variety of G protein-coupled receptors. We have demonstrated the activation of Kir3.1+3.2A channels by Gβγ and by agonist stimulation of Gi/o-coupled receptors but not by Gs-coupled receptors. We present evidence to suggest a key role for the Ga subunit in determining the specificity of coupling between receptor and channel.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection

HEK293 cells (human embryonic kidney cell line) were maintained in minimum essential medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (stocks: 10,000 units/ml penicillin, 1 mg/ml streptomycin), at 37 °C in humidified 95% O₂, 5% CO₂. A lipid-based transfection procedure was used (LipoFectAMINE; Life Technologies, Inc.) and monoclonal cell lines established by picking single colonies of cells following transfection and growth under selective pressure. For the channel-expressing HKIR3.1/3.2 line, 727 µg of G418 (Life Technologies, Inc.) was used and for channel+receptor-expressing lines, we used a dual selection strategy with 727 µg of G418 and 364 µg/ml Zeocin (Invitrogen). For transient expression of receptors, G proteins, and other components, cells were co-transfected with cDNA (100–150 ng) encoding the humanized, red-shifted variant of jellyfish green fluorescent protein (pEGFP-N1; CLONTECH) and cells visualized using a Nikon Diaphot epifluorescent microscope.

Molecular Biology

Standard molecular cloning and mutagenesis techniques were employed throughout. All G protein-coupled receptors and G protein subunits, mutants, and chimeras were cloned into pcDNA1, pcDNA3, or pcDNA3.1/Zeo(+) (Invitrogen). All cDNAs were sequenced to confirm...
their identity. For the expression of Kir3.1 +3.2A, a bicistronic vector was engineered that enabled both subunits to be expressed in the same vector. This vector contained two restriction enzyme polylinkers around a picornavirus internal ribosome entry site (IRES). An IRES-containing plasmid (pIRES1hyg) was obtained from CLONTECH, and the IRES element was amplified using PCR with three additional restriction enzyme sites insertted at the 5’ and 3’ ends using PCR. This fragment was subcloned into pcDNA3. The channel subunits were then subcloned into the newly engineered pcDNA3 and a stable HEK293 cell line established (HKIR3.1/3.2). For some experiments, we made stable cell lines that expressed both channel and receptor. In these instances, receptors (in pcDNA3.1/Zeo+) were transfected into the stable Kir3.1+3.2A line and selected for using a dual selection strategy. Point mutants of Gα1i (C351G and C351I) were made as in Ref. 29. COOH-terminal chimeras between Gα1i and Gαi2 were made using a PCR-based approach with a high fidelity DNA polymerase (Vent; New England Biolabs) and 18–25 cycles. To replace the COOH-terminal 6 amino acids, a single round of PCR was used and appropriate 5’ and 3’ restriction enzyme sites inserted. For the chimera were the COOH-terminal 13, 16, and 20 amino acids of Gα1i were replaced with Gαi2; two rounds of PCR were employed. The first round replaced the COOH-terminal 10, 13, and 17 amino acids, respectively, of Gα1i, and those of Gαi2, and appropriate 5’ restriction enzyme sites while the second round added the remaining 3 amino acids, stop codon, and 3’ restriction enzyme sites.

Biochemistry

Western Blotting—SDS-polyacrylamide gel electrophoresis and transfer of proteins to nitrocellulose was performed using a Bio-Rad minigel system according to the manufacturer’s instructions. Cells were harvested in phosphate-buffered saline and total cell homogenate lysed with an equal volume of 2× gel loading buffer and probed with a primary polyclonal rabbit antibody to Gαi2 (Santa Cruz) and the appropriate secondary antibody before visualization of bands using the ECL Western blot analysis kit (Amer sham Pharmacia Biotech).

Adenylyl Cyclase Assay—For the measurement of A2A and β receptors-mediated accumulation of cAMP, the cell lines HKIR3.1/3.2/ A2A and HKIR3.1/3.2/B1 were grown to 20% confluence in six-well dishes. Bead complex was pregelled with 5 μCi of [3H]adenine/well (in minimum essential medium) overnight at 37 °C and then incubated with adenosphosphate (1 μM for A2A receptors) and the phosphodiesterase inhibitor R02-1724 (100 μM) in serum-free medium, for 30 min at 37 °C. agonist (1 μM NECA for A2A, 10 μM isopropanol for β1) was then added and incubated for 15 min at 37 °C. Medium was then aspirated and cells washed with serum-free medium. Reactions were terminated by the addition of 2.5% perchloric acid and 0.1 mM cAMP at 4 °C. [3H]cAMP was isolated by sequential chromatography using Dowex 50-alumina columns. Each fraction was collected in a scintillation vial containing 5 ml of Ultima Gold MV scintillant (Packard). A Beckman LS6000TA liquid scintillation counter was used to quantitate radioactivity. Reactions were done in triplicate, and data are expressed as percentage conversion of [3H]cAMP to [3H]lucAMP.

Radioligand Binding—Cells were washed and harvested into binding buffer (50 mM Tris-HCl, 1 mM MgCl2, pH 7.4) in the presence of protease inhibitors. They were then homogenized (10 mM Tris-HCl) and put on ice for 15 min, after which time an equal volume of 500 mM sucrose plus 10 mM Tris-HCl was added to restore toxicity. Cells were homogenized using a glass-on-glass Dounce homogenizer. The homogenate was spun at 4 °C and the cell pellet resuspended in binding buffer. Specific binding was assessed by using the radioligands [3H]DPCPX (Axon Instruments) and [8-14C]GTPγS (NEN Life Science Products) in separate vectors (pcDNA3). No inwardly rectifying K+ currents were observed when individual subunits were transfected alone (Fig. 1A), but transfection of both subunits together resulted in the expression of strong inwardly rectifying K+ currents. We subcloned both Kir3.1 and Kir3.2A subunits into a single bicistronic vector around an IRES (Fig. 1B), which allowed the expression of both subunits on a single plasmid. A monoclonal stable line expressing Kir3.1+3.2A (hereafter designated as HKIR3.1/3.2) was then established using this vector, and currents were recorded using the whole cell configuration of the patch clamp technique. The inwardly rectifying K+ currents were blocked by external Ba2+, and an example of these currents and the corresponding current-voltage relationship is shown in Fig. 1 (C and D).

Materials and Drugs

Solutions were as follows (concentrations in mM): pipette solution, 107 KCl, 1.2 MgCl2, 1 CaCl2, 10 EGTA, 5 HEPES, 2 MgATP, 0.3 NaGTP (pH 7.2); bath solution, 140 KCl, 2.6 CaCl2, 1.2 MgCl2, 5 HEPES (pH 7.4). All materials for cell culture were obtained from Life Technologies, Inc, and Invitrogen. Radioligands were purchased from NEN Life Science Products and antibodies from Santa Cruz. Molecular biology reagents were obtained from New England Biolabs or Roche Molecular Biochemicals. All chemicals were from Sigma, Calbiochem, or RBI. Oligonucleotides were from Genosys Biotechnologies. Drugs were made up as concentrated stock solutions and kept at −20 °C or −80 °C.

RESULTS

Characterization of Kir3.1+3.2A Cell Line—Channel-forming subunits, receptors, and G proteins were expressed in a mammalian cell line (HEK293). These cells are electrically silent (in non-transfected cells, current density measured in symmetrical K+ solutions at −100 mV was 9.6 ± 1.4 pA/pF, n = 24) and do not appear to possess significant endogenous inwardly rectifying K+ currents. The native neuronal channel is a heterotetrameric structure thought to comprise both Kir3.1 and Kir3.2 subunits. In initial experiments, the cDNAs encoding Kir3.1 and Kir3.2A were transiently transfected into cells in separate vectors (pcDNA3). No inwardly rectifying K+ currents were observed when individual subunits were transfected alone (Fig. 1A), but transfection of both subunits together resulted in the expression of strong inwardly rectifying K+ currents. We subcloned both Kir3.1 and Kir3.2A subunits into a single bicistronic vector around an IRES (Fig. 1B), which allowed the expression of both subunits on a single plasmid. A monoclonal stable line expressing Kir3.1+3.2A (hereafter designated as HKIR3.1/3.2) was then established using this vector, and currents were recorded using the whole cell configuration of the patch clamp technique. The inwardly rectifying K+ currents were blocked by external Ba2+, and an example of these currents and the corresponding current-voltage relationship is shown in Fig. 1 (C and D).

G Protein Regulation of Kir3.1+3.2A Channels—The family of Kir3.x channels are regulated by heterotrimeric G proteins. To mimic the activation of G proteins, we used the non-hydrolyzable analogue of GTP, GTPγS. Basal currents were potentiated by the inclusion of 500 μM GTPγS in the pipette solution (Fig. 1E). It has been established that free Gβγ subunits but not Gα subunits mediate channel activation through a direct protein-protein interaction between determinants on the Gβγ dimer and cytoplasmic domains on the Kir3.x subunit (18–24, 31). The currents expressed in our monoclonal cell line demonstrate behavior that is consistent with this body of work. It was possible to transiently transfect the HKIR3.1/3.2 line and identify transfected cells after co-transfection with green fluorescent protein using epifluorescence. Transient transfection of exogenous βγ subunits significantly increased basal current density, whereas overexpression of Gα subunits from Gα11 and Gα2 significantly reduced basal current densities (Fig. 1E). These data confirm that Gβγ activates the channel, while the reduction in current density by overexpression of α subunits is
Potentiation of Kir3.1+3.2A Currents by Gi/o-coupled Receptors—We next investigated the issue of receptor specificity of channel activation by transiently transfecting a number of Gi/o-coupled receptors. A1, A2A, β1, and D1, was unable to significantly activate currents except for a small increase in current density observed with stimulation of the β1 adrenergic receptor (Fig. 2B, lower panel). Interestingly this effect was PTx-sensitive, suggesting that β1 can also couple to Gβγ proteins to activate the channel. PTx treatment to inhibit any Gβγ proteins that might be competing for Gβγ combined with the overexpression of Gαi still did not allow the Gβγ-coupled receptors to activate the Kir3.1+3.2A currents (Fig. 2C).

To ascertain that this observation of specificity of receptor coupling was not an anomaly of the expression system, for example, due to stronger or more efficient transient expression of Gβγ-coupled receptors than Gαi-coupled receptors, stable monolayer cell lines were established that expressed both channel subunits and a receptor (A1, denoted as HKIR3.1/3.2/A1, A2A, β1, or β2, denoted as HKIR3.1/3.2/B1). Mean basal current density in these cell lines was no different to that measured in HKIR3.1/3.2 after transient transfection of receptors. Similarly to the responses seen in the transiently transfected cells, only the stable lines ex-
pressing Gα-coupled receptors (i.e. A1 and a2A) were able to potentiate Kir3.1+3.2A currents (data not shown).

Confirmation of Expression of Functional Receptors Coupled to an Intact Second Messenger System—The lack of potentiation of currents by stimulation of Gαs-coupled receptors was not due to low expression levels of Gαs since overexpression, together with relevant receptor in PTx-treated cells, did not significantly enhance currents (Fig. 2C). In order to confirm that these cells express endogenous Gαs, we performed Western blots on total cell homogenate from the HKIR3.1/3.2/A1 and HKIR3.1/3.2/A2A lines. Clearly, the HKIR3.1/3.2/A1 line expressed Gαs. In the HKIR3.1/3.2/A2A line, expression of Gαs was lower but was revealed upon longer exposure of the blot. Transfection of the Gαs-containing plasmid led to increased protein expression (Fig. 3A).

It is clear that the Gαs-coupled receptors were functionally expressed since K+ currents were potentiated by stimulation of these receptors. However, since no potentiation was observed with the Gβγ-coupled receptors, it was important to determine that these receptors were actually being expressed at the cell membrane and were functionally coupled to downstream second messenger pathways.

We investigated the biochemical characteristics of the monoclonal lines HKIR3.1/3.2/A1, HKIR3.1/3.2/A2A, and HKIR3.1/3.2/B1. Equilibrium radioligand binding experiments using [3H]DPCPX (A1) and [3H]CGS21680 (a2A) were performed on homogenates from the HKIR3.1/3.2/A1 and HKIR3.1/3.2/A2A stable lines. Non-transfected cells did not exhibit binding of either radioligand (data not shown). Both HKIR3.1/3.2/A1 and HKIR3.1/3.2/A2A lines exhibited saturation of binding of appropriate ligand (Fig. 3B). It was noted that the A2A receptor was stably expressed at lower levels than the A1 receptor; this is an observation that has also been made in CHO cells (32).

To confirm signaling to a downstream effector, namely adenylate cyclase, agonist-induced cAMP accumulation was measured in the HKIR3.1/3.2/A2A and HKIR3.1/3.2/B1 lines. A large increase in cAMP levels was observed in both lines in response to receptor stimulation (Fig. 3C).

Coupling of Channels to PTx-insensitive G Protein Mutants—Thus far, we report that, as in native tissue, Kir3.1+3.2A currents were strongly activated by Gβγ but not Gα subunits and by stimulation of Gαs- but not Gαγ-coupled receptors. To further investigate this specificity of channel activation, we took advantage of the PTx sensitivity of the Gαs family of G proteins. These G proteins have a conserved cysteine residue in the α subunit 4 amino acids from the COOH terminus, which is ADP-ribosylated by PTx (33). Mutation of this residue prevents modification by PTx, thus resulting in a PTx-insensitive G

![Fig. 2. Agonist stimulation of Gαγ-coupled receptors leads to the enhancement of Kir3.1+3.2A currents. A, activation of A1 receptors by 1 μM NECA increased Kir3.1+3.2A currents. Currents were elicited as illustrated in Fig. 1A. This shows the effects of 1 μM NECA on Kir3.1+3.2A currents. The left panel shows control, the middle panel is in the presence of 1 μM NECA, and the right panel is after washing NECA off. B, summary of the effects of stimulating three pairs of G protein-coupled receptors. The upper panel shows data for Gαγ-coupled receptors and the lower panel shows data for Gαs-coupled receptors. Maximal concentrations of agonists (1 μM NECA (A1 and A2A), 3 μM noradrenaline (α2A), 10 μM isoprenaline (β1), 10 μM quinpirole (D2S), and 1 μM SKF38393 (D1)) were applied for 20–40 s. For PTx experiments, cells were exposed to PTx (100 ng/ml), post-transfection, for at least 16 h. C, Kir3.1+3.2A currents were not activated by Gαs-coupled receptors, even in the presence of excess Gαs. Cells were transfected with receptor and Gαs and then treated with PTx.](image-url)
nM and A2A (HKIR3.1/3.2/A2A line) stable lines compared with a control protein expression of Gs-coupled receptors. 1

a receptor and mutant Gα reduced basal current density similarly to wild type Gα. This did not enhance membrane currents and in fact significantly

activation via A2A and C exhibit any specific binding (not shown). Data were pooled from at least

a protein (34, 35). Gα was transienly transfected. B, [3H]DPCPX and [3H]CGS21680 saturation binding to A1 (open circles) and A2A receptors (solid circles) in the HKIR3.1/3.2/A1 and HKIR3.1/3.2/A2/A2A cell lines. The following binding parameters were obtained: A1, Kd = 0.37 ± 0.06 nM and Bmax = 5.67 ± 0.33 pmol/mg protein; A2A, Kd = 13.5 ± 6.3 nM and Bmax = 4.23 ± 0.54 pmol/mg protein. Non-transfected cells did not exhibit any specific binding (not shown). Data were pooled from at least six experiments, with each counted in triplicate. C, adenylate cyclase activation via A2A and β1 receptors in lines HKIR3.1/3.2/A2A and HKIR3.1/3.2/A2/B1, respectively. Total [3H]cAMP conversion was measured as described under “Experimental Procedures.” A2A receptors were stimulated for 15 min by 1 μM NECA and β1 receptors by 10 μM isoprorenaline. Data are from four to six experiments, each being performed in triplicate.

protein (34, 35). Gα1 was mutated at this position to glycine or isoleucine (29). After transiently transfecting receptors and mutant Gα1 subunits into the HKIR3.1/3.2 line, cells were treated with PTX to preclude coupling between receptor and endogenous Gα1, but still allow the study of coupling between receptor and mutant Gα1. Expression of these mutants alone did not enhance membrane currents and in fact significantly reduced basal current density similarly to wild type Gα1 (Fig. 1E) and in PTX-treated cells, stimulation of any of the Gα1-coupled receptors tested were unable to enhance Kir3.1+3.2A currents (see Fig. 2B). When the mutant Gα11 subunits, Gα13, C351G and Gα13, C351I, were co-expressed, agonist stimulation of receptor led to a large enhancement of currents (Fig. 4A). This was observed with both A1 (Fig. 4B) and A2A (Fig. 4C) receptors. In analogous experiments, expression of Gα1 in PTX-treated cells did not support coupling between Kir3.1+3.2A and any receptors (Figs. 2C and 4B). Thus, the PTX-insensitive Gα1 subunits were able to rescue signaling between Gα1-coupled receptors and Kir3.1+3.2A in PTX-treated cells.

A COOH-terminal Chimera between Gα1 and Gα Completely Swaps Coupling between Receptor and Kir3.1+3.2A Channels—Our data suggest that Gα is important in the determination of receptor selectivity of channel activation. This hypothesis was further investigated by the use of chimeric G proteins. Since the COOH-terminal 20 amino acids of G protein α subunits are implicated in the selectivity of interactions with G protein-coupled receptors (36), we made a series of COOH-terminal chimeras between the G proteins Gα1 and Gα, where between 6 and 20 amino acids of Gα1 were replaced with the corresponding residues of Gα. These experiments were done in PTX-treated cells to prevent interaction of receptor with endogenous Gα1, and the data are shown in Fig. 5. Strikingly, a 13-amino acid chimera (Gα i/o13) allowed a complete swap of receptor coupling; A2A receptor stimulation by NECA strongly enhanced currents to a similar level observed with A1 receptor stimulation under control (non-PTX-treated) conditions (Fig. 5A). Further replacements, Gα i/o16 and Gα i/o20, had similar, although not as profound, effects (Fig. 5B). The effects of the Gα i/o13 chimera were not unique to the A1/A2A receptors; this chimera was also able to swap receptor/channel coupling from the A2A to the β1 adrenergic receptor (Fig. 5C). Thus, we have managed to potentiate the Kir3.1+3.2A currents via Gα-coupled receptors by using chimeric G proteins between Gα11 and Gαα.

Disruption of Coupling between the A1 Receptor and Kir3.1+3.2A by a COOH-terminal Chimera between Gα1 and Gα, Further evidence that supports a key role for the G protein α subunit is a dominant negative effect that we observed with a COOH-terminal chimera between Gα1 and Gα,; this chimera is mainly Gα1, but has the COOH-terminal 13 amino acids of Gα11 (Gα i/o13). This acted to significantly disrupt coupling between the A1 receptor and Kir3.1+3.2A (a reduction of approximately 70%; Fig. 6). NECA-induced currents in control cells were 217.0 ± 38.9 pA/pF (n = 15), whereas in cells where Gα i/o13 was expressed, induced currents were significantly reduced: 68.6 ± 21.3 pA/pF (n = 20, p = 0.045). This dominant negative effect is likely to be specific, as over-expression of Gα with the A1 receptor did not suppress basal current (Fig. 4B).

DISCUSSION

Reconstitution of Specificity of Activation of Kir3.1+3.2A Channels—One of the aims of this study was to examine whether the specificity of Kir3.1+3.2A channel activation by G protein-coupled receptors, widely observed in native tissue such as neuronal and atrial cells, could be reconstituted in an heterologous expression system. By making a stable channel-expressing mammalian cell line (HKIR3.1/3.2), we were able to reconstitute the specificity of channel activation. We have shown that only Gα1-coupled receptors, but not Gβγ-coupled receptors, activate Kir3.1+3.2A channels in our HEK293 cell line. Even overexpression of Gα1 did not lead to current enhancement through Gα1-coupled receptors, either in PTX-treated or non-treated cells, suggesting that Gα is not involved in the activation of these channels. Thus, in our system, Gβγ dimers derived from inhibitory heterotrimeric G proteins are able to activate Kir3.1+3.2A heteromultimeric currents while the stimulatory Gα is not.
A number of studies have demonstrated that, under given conditions, it is possible to activate Kir3.x or native currents through Gs-coupled receptors (37–39). However, some issues arise in relation to these and our work. Often a single receptor pathway has been used. Ruiz-Velasco and Ikeda (38) found they could activate Kir3.1 and Kir3.4 channels heterologously expressed in superior cervical ganglion neurons through the vasoactive intestinal peptide (VIP) receptor. VIP does not exclusively stimulate Gs-coupled receptors: it has been shown to couple to Gαi1/2 (40) and also to elevate intracellular Ca2+ levels (41). The VIP-mediated effects were enhanced by pre-treatment with PTx, leading to the authors’ suggestion that the uncoupling of PTx-sensitive G proteins allowed more PTx-insensitive G proteins to activate the channels. We found that PTx had no effects on the inability of Gs-coupled receptors to activate these channels and did not observe channel activation by Gs-coupled receptors either in the presence or absence of PTx. Lim et al. (37) found potentiation of currents through the β2 adrenergic receptor but it is now appreciated that this receptor is able to couple to Gi (42, 43). Promiscuous coupling of receptors to more than one G protein is apparent with many receptors (44–47). One explanation advanced for the specificity of coupling is that there is more Gi/o than Gs in most cells; indeed, in the above studies, it was often necessary to overexpress GaS and in some instances the methods used to elevate GaS were not direct. Sorota et al. (39) used infection with an adenovirus, and this may have nonspecific and toxic effects on the cells. In our hands, even the overexpression of GaS did not lead to coupling.

Specificity of coupling may not be an absolute phenomenon and may depend on the relative levels of expression of channel, G protein, and receptor. Even though the current densities in our heterologous system are higher than seen in native neurons, we are still able to demonstrate an essentially absolute selectivity mechanism more analogous to that seen in native tissue. In the studies detailed above, no direct comparison is made with a Gi/o-coupled pathway under equivalent conditions. Indeed, in the work of Sorota et al. (39) on the native current in atrial myocytes, the levels of current activation through the β adrenergic receptor are lower than that seen through the muscarinic receptor, even after the overexpression of GaS.

**A Key Role for the Ga Subunit in Determining Specificity of Channel Activation**—We have three lines of evidence that the Ga subunit is the key determinant of selectivity of channel activation by Gi/o-linked heptahelial receptors. First, the transfection of PTx-resistant mutants of GaS was able to rescue coupling between Gi/o-coupled receptors and Kir3.1+3.2A, but overexpression of GaS with Gs-coupled receptors under similar conditions was not able to do so. Second and most tellingly, it was possible to swap coupling between these families of receptor by exchanging only a few amino acids on the COOH terminus of Gi with those of Gs. Finally, we were able to disrupt...
coupling between a Gi/o-coupled receptor (A1) and Kir3.1-3.2A by using a Ga$i_1$ chimera that contained the COOH-terminal 13 amino acids of Ga$i_1$. A similar strategy was used by Gilchrist et al. (48), who constructed “minigenes” of the COOH-terminal 11 amino acids of Ga$i_1$ and showed selective disruption of M2 muscarinic receptor coupling to Kir3.1-3.4.

Schreibmayer et al. (49) showed inhibition of Gβγ1γ2-induced currents by activated Ga$i_1$, but not Ga$i_2$ or Ga$i_3$. In contrast, we have found that Ga subunits inhibit basal (non-agonist-stimulated) currents (presumably due to sequestration of free βγ subunits) but that PTx-insensitive mutants of Ga$i_1$ actually supported coupling between receptor and channel. However, there are a number of methodological differences in expression system and experimental design; for example, our studies are performed in the whole cell configuration, while those studies were largely performed in excised patches in the inside-out configuration.

Expression in PTx-treated cells of a COOH-terminal chimera, in which 13 amino acids of Ga$i_1$ were replaced with the corresponding residues of Ga$s$, swaps the receptor coupling profile, i.e. Ga$-$coupled receptors can now stimulate the channel whereas Ga$s$-coupled receptors cannot. With 16 and 20 amino acid replacements, a swap of coupling was still observed, although it was not quite so profound. This apparent “drop-off” could simply be due to less efficient protein folding of these chimeras, and a similar finding was observed by Conklin et al. (50). Several investigators have used a chimeric G protein approach to alter the fidelity of receptor activation, i.e. receptors not normally coupled to a particular class of G protein then become able to stimulate that G protein (50–53). It has been shown that substitution of as few as 4 amino acids can alter receptor signaling to G proteins (50). Replacing the COOH-terminal 4 amino acids of Gq with those of Ga$i_2$ allowed the stimulation of phospholipase C by D2 dopaminergic and A1 adenosine receptors, which normally couple exclusively to Ga$i$. However, whether a swap of coupling occurred was not established, i.e. that Gq-coupled receptors are no longer able to stimulate phospholipase C activity with the Gβγ/Ga$i_1$ chimera. Our data suggest that it is possible with some receptor groups to achieve a clean switch by COOH-terminal replacement. In addition, the carboxyl terminus of Ga subunits may not be the sole determinant of mediating specificity as other regions have been implicated in receptor interactions, notably at the NH2 terminus (36, 54). It is likely that the extent of the involvement of the COOH terminus and other regions of the Ga subunits varies with receptor; indeed, this was found by Conklin et al. (50) with the A1 and D2 receptors and their coupling to Gβγ/Ga$i$ chimeras.

Our data demonstrate that we can reconstitute specificity of coupling between seven helix receptors and Kir3.1-3.2A channels. Basal currents are enhanced by the overexpression of the Gβγ dimer but not the Ga subunit. The pivotal role of the Gβγ dimer in channel activation is not being contested. We have no
evidence of channel activation via G<sub>q</sub>-coupled receptors, even when PTX-sensitive G proteins are uncoupled and/or G<sub>q</sub> is overexpressed. By using single point mutations of G<sub>q</sub>, we can still activate the channel via G<sub>q</sub>-coupled receptors in PTX-treated cells and by making chimeras between G<sub>q1</sub> and G<sub>q2</sub>, we can swap coupling from G<sub>i/o</sub>- to G<sub>s</sub>-coupled receptors and Kir3.1+3.2A channel. This shows a schematic of the GsGi13 chimera, together with a bar chart indicating current density measured in response to stimulation of the A<sub>1</sub> receptor by 1 μM NECA under control conditions, and with the co-expression of GsGi13.

Do similar considerations apply to other effectors where Gβγ subunits are the direct mediators such as the inhibition of voltage-gated calcium channels of the N and P/Q type (55–58)? It is apparent that calcium channel inhibition can be mediated through a number of receptors coupling to a number of different families of G proteins so the mechanisms may be different (59, 60).

The molecular mechanisms underlying receptor specificity of Kir<sub>3.x</sub> activation are largely unknown. It has been proposed without strong supportive evidence that there is compartmentalization of signaling components: either between receptor and channel, G protein and channel, or all three. Our data suggest a key role for the α subunit and seem to make it unlikely that there is compartmentalization between receptor and channel as receptor selectivity can be switched with G<sub>q</sub>-α<sub>q</sub> chimeras. So what might the possible relationship be between the α subunit and the channel complex? This could be via a direct protein-protein interaction or indirectly involving other accessory proteins. The heterotricmeric G protein has been found to bind to the NH<sub>2</sub>-terminus of Kir3.1 (18, 61), but whether G<sub>q</sub>-containing heterotrimers were preferentially bound in comparison to G<sub>q</sub>-containing heterotrimers was not addressed. Our data are an important insight in understanding how receptor selectivity is achieved in a Gβγ-activated system and provides a basis for future mechanistic studies.

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46. Cotecchia, S., Koblika, B. K., Daniel, K. W., Nolan, R. D., Lapetina, E. Y., Caron, M. G., Lefkowitz, R. J., and Regan, J. W. (1990) J. Biol. Chem. 265, 63–69
47. Chabre, O., Conklin, B. R., Brandon, S., Bourne, H. R., and Limbird, L. E. (1994) J. Biol. Chem. 269, 5730–5734
48. Gilchrist, A., Bunemann, M., Li, A., Hosey, M., and Hamm, H. E. (1999) J. Biol. Chem. 274, 6610–6616
49. Schreibmayer, W., Dessauer, C. W., Vorobiov, D., Gilman, A. G., Lester, H. A., Davidson, N., and Dascal, N. (1996) Nature 380, 624–627
50. Conklin, B. R., Farfel, Z., Lustig, K. D., Julius, D., and Bourne, H. R. (1993) Nature 363, 274–276
51. Conklin, B. R., Herzmark, P., Ishida, S., Veyno-Yasenetskaya, T. A., Sun, Y., Farfel, Z., and Bourne, H. R. (1996) Mol. Pharmacol. 50, 885–890
52. Tsu, R. C., Ho, M. K. C., Yung, L. Y., Joshi, S., and Wong, Y. H. (1997) Mol. Pharmacol. 52, 38–45
53. Fong, C. W., Bahia, D. S., Rees, S., and Milligan, G. (1998) Mol. Pharmacol. 54, 249–257
54. Kostenis, E., Degtyarev, M. Y., Conklin, B. R., and Wess, J. (1997) J. Biol. Chem. 272, 19107–19110
55. Herlitze, S., Garcia, D. E., Mackie, K., Hille, B., Scheur, T., and Catterall, W. (1996) Nature 380, 258–262
56. Ikeda, S. R. (1996) Nature 380, 255–258
57. Dolphin, A. C. (1998) J. Physiol. 506, 3–11
58. Zamponi, G. W., and Snutch, T. P. (1998) Curr. Opin. Neurobiol. 8, 351–356
59. Delmas, P., Abogadie, F. C., Dayrell, M., Haley, J. E., Milligan, G., Caulfield, M. P., Brown, D. A., and Buckley, N. J. (1998) Eur. J. Neurosci. 10, 1654–1666
60. Jeong, S.-W., and Ikeda, S. R. (1999) J. Neurosci. 19, 4755–4761
61. Slesinger, P. A., Reuveny, E., Jan, Y. N., and Jan, L. Y. (1995) Neuron 15, 1145–1156