Effective information transfer from the $\alpha_{1b}$-adrenoceptor to $G\alpha_{11}$ requires both $\beta/\gamma$ interactions and an aromatic group four amino acids from the C-terminus of the $G$ protein

Sen Liu, Juan J. Carrillo, John D. Pediani and Graeme Milligan

Molecular Pharmacology Group
Division of Biochemistry and Molecular Biology
Institute of Biomedical and Life Sciences
University of Glasgow
Glasgow G12 8QQ
Scotland, U.K.

All correspondence to
Graeme Milligan
Davidson Building
University of Glasgow
Glasgow G12 8QQ
Scotland, U.K.

Tel: 44 141 330 5557
FAX: 44 141 330 4620
e-mail: g.milligan@bio.gla.ac.uk

Running title: Receptor activation of $G_{11}$. 
Co-expression of the α₁b-adrenoceptor and Gα₁₁ in cells derived from a Gα₁/Gα₁₁ knock out mouse allows agonist-mediated elevation of intracellular Ca²⁺ levels that is transduced by βγ released from the G protein α subunit. Mutation of Tyr³⁵⁶ of Gα₁₁ to Phe, within a receptor contact domain, had little effect on function but this was reduced greatly by alteration to Ser and virtually eliminated by conversion to Asp. This pattern was replicated following incorporation of each form of Gα₁₁ into fusion proteins with the α₁b-adrenoceptor. Following a [³⁵S]GTPγS binding assay, immunoprecipitation of the wild type α₁b-adrenoceptor-Gα₁₁ fusion protein indicated that the agonist phenylephrine stimulated guanine nucleotide exchange on Gα₁₁ more than 30 fold. Information transfer by agonist was controlled in residue³⁵⁶ Gα₁₁ mutants with rank order Tyr>Phe>Trp>Ile=Ala=Gln=Arg>Ser>Asp although these alterations did not alter the binding affinity of either phenylephrine or an antagonist ligand. Mutation of a βγ contact interface in the α₁b-adrenoceptor-Tyr³⁵⁶ Gα₁₁ fusion protein did not alter ligand binding affinity but did reduce greatly βγ binding and phenylephrine-stimulation of [³⁵S]GTPγS binding. It also prevented agonist elevation of intracellular Ca²⁺ levels, as did a mutation in Gα₁₁ that prevents G protein subunit dissociation. These results indicate that a bulky aromatic group is required four amino acids from the C-terminus of Gα₁₁ to maximise information transfer from an agonist-occupied receptor and disprove the hypothesis that tyrosine phosphorylation of this residue is required for G protein activation (Umemori, H., Inoue, T., Kume, S., Sekiyama, N., Nagao, M., Itoh, H., Nakanishi, S., Mikoshiba, K. and Yamamoto, T. (1997) Science 276, 1878-1881). This is distinct from Gα₁i, where hydrophobicity of the amino acid is the key determinant at this location. They also further demonstrate a key role for the βγ complex in enhancing receptor to G protein α subunit information transfer.
Information transfer between G protein-coupled receptors (GPCRs) and G protein α subunits involves the induced release of GDP from the nucleotide binding site of the G protein and its subsequent replacement by GTP [1]. This is often studied by monitoring the binding of an analogue of GTP, [35S]GTPγS, that is resistant to the inbuilt GTPase activity of the G protein α subunit [2-3]. Receptor-mediated activation of phospholipase Cβ1 is mediated via members of the Gq-family of G proteins [4-6]. Resistance of these G proteins to ADP-ribosylation by bacterial toxins and their low rates of guanine nucleotide exchange made their initial purification and characterization an extremely difficult task [7-9]. This second feature has also limited efforts to use the binding of [35S]GTPγS to monitor their activation. In contrast, the ease of use of this assay for members of the pertussis toxin-sensitive Gi-family of G proteins has resulted in its widespread application [2-3].

The pertussis toxin-sensitive G proteins have a conserved Cys located 4 amino acids from the C-terminus that is the site of ADP-ribosylation by the toxin. As this modification prevents GPCR-mediated activation of these G proteins it provided early compelling evidence of a key role for the extreme C-terminal region of G protein α subunits in productive interactions with GPCRs [10]. Recently, extensive mutagenesis at this site in both Gαi1 [11] and Gαi3 [12] has indicated that the Cys is not essential for information transfer and that the effectiveness of receptor-mediated activation is determined by the hydrophobicity of the residue at this position.

The widely expressed Gq-family G proteins, Gαq and Gα11, share an identical C-terminal decapeptide with Tyr356 located 4 amino acids from the C-terminus of Gα11 [4]. Previous studies have suggested that this Tyr can become phosphorylated in response to GPCR activation and that this may be a key event in activation of the G protein [13]. We have recently employed an immunoprecipitation strategy in concert with a [35S]GTPγS binding assay to monitor directly GPCR and agonist-induced guanine nucleotide exchange on Gα11 [14]. Herein we extend this approach to analyse the effects on information transfer between the α1b-adrenoceptor and forms of Gα11 in which Tyr356 was altered to a range of amino acids. There is no inherent requirement for Tyr at this position and thus for its potential phosphorylation in the
activation of the G protein. However, as Phe and Trp are the other amino acids that can substitute effectively a key role for a bulky aromatic group is evident.

Although the guanine nucleotide binding site and GTPase machinery are defined by the G protein α subunit, the β/γ complex plays a key role, with growing evidence for a direct role of the γ subunit in contacting the receptor to enhance guanine nucleotide exchange [15-16]. By mutating a key β/γ contact site in Gα11 we also provide support for this notion because the modified α subunit exchanges guanine nucleotide significantly less effectively in response to receptor activation that does the wild type. Overexpression of β/γ with a fusion protein between the α1b-adrenoceptor and the mutant form of Gα11 with reduced β/γ binding affinity enhances the effectiveness of agonist-stimulated guanine nucleotide exchange.

Materials and Methods

Materials

A fibroblast cell line (EF88) [17-19] derived from a combined Gαq/Gα11 double knockout mouse [20-21] was the gift of Dr. M.I. Simon, California Institute of Technology, Pasadena CA. [3H]Prazosin and [35S]GTPγS were purchased from NEN Life Science Products. Dulbecco’s modified Eagle’s medium (DMEM), newborn calf serum, L-glutamine and trypsin/ethylenediamine tetra-acetic acid were purchased from Life Technologies Inc. (Paisley, Scotland). Fura 2-AM, phentolamine HCl, phenotolamine, 4-(2-hydroxyethyl)-1-piperazine sulphonic acid (HEPES) and ethylene glycol-bis(β-aminoethylether)-N,N,N’, N’-tetraacetic acid (EGTA) were purchased from Sigma (Dorset, UK). CQ (C-terminus of Gq) antisera have previously been described [22-23]. All other chemicals were from sources described in Stevens et al. [14, 24].

G protein mutations and construction of fusion proteins

Residue356 of Gα11 (wild type is Tyr) was converted to a range of other amino acids. The Ile25Ala, Glu26Ala (IE) mutant was constructed based on the studies of Evanko et al.on Gαq [25]. These modified
forms of G\(\alpha_{11}\) were also incorporated into \(\alpha_{1b}\)-adrenocceptor-G\(\alpha_{11}\) fusions proteins as described previously [14]. Each was fully sequenced before its expression and analysis.

**Transient Transfection of HEK293 cells**

HEK293 cells were maintained in DMEM supplemented with 0.292 g/liter L-glutamine and 10% (v/v) newborn calf serum at 37°C in a 5% CO\(_2\) humidified atmosphere. Cells were grown to 60-80% confluency before transient transfection in 60mm dishes. Transfection was performed using LipofectAMINE reagent (Life Technologies, Inc.) according to the manufacturer’s instructions.

**[Ca\(^{2+}\)]\(_i\) measurement: Cell culture and transfection**

EF88 are a fibroblast cell line, derived from the embryos of mice in which expression of the \(\alpha\) subunits of both G\(_q\) and G\(_{11}\) had been eliminated by targeted gene disruption [17-19]. These were grown in DMEM supplemented with 10% (v/v) heat inactivated foetal bovine serum and L-glutamine (1 mM) in a 95% air and 5% CO\(_2\) atmosphere at 37°C. A portion of the cells harvested during trypsinization were plated on to glass coverslips and after a 24 h growth period they were transfected using LipofectAMINE (Life Technologies Inc.) according to the manufacturers’ instructions. After 3 h cells were washed twice with OPTIMEM 1 and then cultured in DMEM growth medium for a further 24 h. A total of 3 \(\mu\)g of pCDNA3 containing the relevant cDNA species were used to transfect each coverslip.

**[Ca\(^{2+}\)]\(_i\) imaging**

Transfected cells were loaded with the Ca\(^{2+}\)-sensitive dye Fura-2 by incubation (15-20 min, 37°C) under reduced light in DMEM growth medium containing the dye’s membrane-permeant acetoxyxymethylester form (1.5 \(\mu\)M). Loaded cells were illuminated with an ultra high point intensity 75 W xenon arc lamp (Optosource, Cairn Research, Faversham, Kent, UK) and subsequently imaged using a Nikon Diaphot inverted microscope equipped with a Nikon 40 x oil immersion Fluor objective lens (NA=1.3) and a monochromator (Optoscan, Cairn Research, Faversham, Kent, UK) which was used to alternate the excitation wavelength between 340/380 nm and to control the excitation bandpass (340 nm band pass =10 nm; 380 nm band pass =8 nm). Fura-2 fluorescence emission at 510 nm was monitored using a high
resolution interline-transfer cooled digital CCD camera, (Cool Snap-HQ, Roper Scientific/Photometrics, Tucson, Arizona). MetaFluor imaging software (version 4.6.8, Universal Imaging Corp., Downing, PA, USA) was used for control of the monochromator, CCD camera and for processing of the cell image data. Sequential images (2 x2 binning) were collected every 2 sec, exposure to excitation light was 100 msec/image and all experiments were undertaken in the absence of extracellular Ca\(^{2+}\) in saline solution comprising: 130 mM NaCl, 5 mM KCl, 1 mM MgCl\(_2\), 20 mM HEPES, 10mM D-glucose, 0.01 mM EGTA, pH adjusted to 7.4 using NaOH.

\([\text{Ca}^{2+}]_i\) image analysis

Ratio images were presented in MetaFluor intensity-modulated display (IMD) mode [26] which associates the colour hue with the excitation ratio value and the intensity of each hue with the source image brightness. Briefly background subtracted images acquired at 340 and 380 nm excitation were first used for calculating the 340/380 nm ratio of each pixel. After determination of the upper and lower thresholds, the ratio value of each pixel was associated with one of the 24 hues from blue (low \([\text{Ca}^{2+}]_i\)) to red (high \([\text{Ca}^{2+}]_i\)). Pooled average IMD ratio intensity values measured from single cells were expressed as the mean ± s. e. mean of at least 10 cells with the vertical lines representing s. e. of mean.

\[^{35}\text{S}]\text{GTP}[\gamma]\text{S binding}\)

\[^{35}\text{S}]\text{GTP}[\gamma]\text{S binding experiments were initiated by the addition of membranes containing 100 fmol of the fusion constructs to an assay buffer (20 mM HEPES (pH 7.4), 3 mM MgCl\(_2\), 100 mM NaCl, 1 µM guanosine 5'-diphosphate, 0.2 mM ascorbic acid, 50 nCi \[^{35}\text{S}]\text{GTP}[\gamma]\text{S}) containing the indicated concentrations of receptor ligands. Non-specific binding was determined in the same conditions but in the presence of 100 µM GTP[\gamma]S. Reactions were incubated for 15 min at 30°C and were terminated by the addition of 0.5 ml of ice cold buffer, containing 20 mM HEPES (pH 7.4), 3 mM MgCl\(_2\) and 100 mM NaCl. The samples were centrifuged at 16,000g for 15 min at 4°C, and the resulting pellets were resuspended in solubilization buffer (100 mM Tris, 200 mM NaCl, 1 mM EDTA, 1.25% Nonidet P-40) plus 0.2% sodium dodecysulfate. Samples were precleared with Pansorbin (Calbiochem), followed by immunoprecipitation with CQ antiserum [22-23] or an antiserum against the N-terminal section of the \(\alpha_{1b}\)-adrenoceptor. Finally,
the immunocomplexes were washed twice with solubilization buffer, and bound $[^{35}\text{S}]$GTP$\gamma$S estimated by liquid-scintillation spectrometry.

$[^{3}\text{H}]$Prazosin binding studies

Binding assays were initiated by the addition of 3 µg of cell membranes to an assay buffer (50 mM Tris-HCl, 100 mM NaCl, 3mM MgCl$_2$, pH 7.4) containing $[^{3}\text{H}]$prazosin (0.05-5 nM in saturation assays and 1.0 nM for competition assays), in the absence or presence of increasing concentrations of phenylephrine. Non-specific binding was determined in the presence of 100µM phentolamine. Reactions were incubated for 30 min at 30°C, and bound ligand separated from free by vacuum filtration through GF/B filters. The filters were washed twice with assay buffer, and bound ligand was estimated by liquid scintillation spectrometry.

Immunoprecipitation and immunodetection studies.

For immunoprecipitations cells were washed once with ice-cold phosphate buffered saline (PBS) and immediately homogenized in a lysis medium, containing 50 mM Hepes pH 7.4, 10mM Na$_4$P$_2$O$_7$, 100 mM NaF, 10 mM EDTA, 0.2 mM Na$_3$VO$_4$, 1% Triton X-100 and a protease inhibitor cocktail (Complete, Roche). Cell lysates were centrifuged (15 min, 13000 rpm) and the supernatants precleared for 1 h with non-specific serum and protein A. Next, samples were incubated overnight with the appropriate antiserum. The immunocomplexes were then captured with protein A-agarose.

For immunoblotting, cell lysates or immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred to PVDF membranes and blocked for 2 h with 5% non fat dried milk in 0.05% Tween 20/Tris buffered saline (TTBS). Then, the PVDF membranes were probed overnight at 4°C with the appropriate antiserum and washed with TTBS. Membranes were incubated for 20 min with horseradish peroxidase conjugated to anti-rabbit IgG (1:20000) (Amersham). Finally, membranes were washed with TTBS and developed by enhanced chemiluminescence.
Results

Co-expression of the $\alpha_{1b}$-adrenoceptor and $\alpha_{11}$ is required to elevate intracellular $[Ca^{2+}]$ in response to the $\alpha_1$-adrenoceptor agonist phenylephrine in cells (EF88) derived from a combined $\alpha_\text{q}$ and $\alpha_{11}$ knock-out mouse (Figure 1 and [14]). Because this effect is blocked by co-expression of $\alpha$ transducin (Figure 1), which is an effective $\beta/\gamma$ sequestering agent, the response to agonist reflects activation of the $G$ protein, the release of $\beta/\gamma$ complex from the $\alpha$ subunit and regulation of a $\beta/\gamma$ sensitive isoform of phospholipase $C[\beta]$. Co-expression of the $\alpha_1$-adrenoceptor with $\text{Tyr}^{356}\text{Asp}\alpha_{11}$ did not result in a significant elevation of intracellular $Ca^{2+}$ (Figure 1). These results indicate an important role for this residue in the $G$ protein. When the $\alpha_{1b}$-adrenoceptor was co-expressed with $\text{Tyr}^{356}\text{Phe}\alpha_{11}$ the response to phenylephrine was only slightly lower and more delayed kinetically than with wild type $\alpha_{11}$ (Figure 1).

Such results negate the suggestion that phosphorylation of $\text{Tyr}^{356}\alpha_{11}$ is required for its activation [13]. $\text{Tyr}^{356}\text{Ser}\alpha_{11}$ was also capable of being activated by the $\alpha_{1b}$-adrenoceptor but response was substantially lower and kinetically substantially slower than with wild type or $\text{Tyr}^{356}\text{Phe}\alpha_{11}$ (Figure 1).

We have recently demonstrated that a fusion protein in which the N-terminus of wild type $\alpha_{11}$ is linked to the C-terminal tail of the $\alpha_{1b}$-adrenoceptor from which the stop codon was eliminated also releases $\beta/\gamma$ complex and elevates $Ca^{2+}$ levels in EF88 cells upon agonist stimulation [14]. Fusion proteins were thus also constructed between the $\alpha_{1b}$-adrenoceptor and each of $\text{Tyr}^{356}\text{Phe}\alpha_{11}$, $\text{Tyr}^{356}\text{Ser}\alpha_{11}$ and $\text{Tyr}^{356}\text{Asp}\alpha_{11}$. Each of these fusion proteins was expressed in EF88 cells and the ability of phenylephrine to elevate intracellular $Ca^{2+}$ measured. These studies recapitulated the co-expression data. Virtually no signal was generated from the fusion containing $\text{Tyr}^{356}\text{Asp}\alpha_{11}$, a poor signal was obtained from the $\text{Tyr}^{356}\text{Ser}\alpha_{11}$ containing version and similar and significantly greater signals were obtained from the fusion proteins containing the $\text{Tyr}^{356}\text{Phe}$ or wild type $\alpha_{11}$ (Figure 2). Again, the signals obtained with the Phe and Ser mutants were delayed kinetically compared to those from the construct containing the wild type $G$ protein (Figure 2). As reported previously [14] co-expression of $\alpha$ transducin fully attenuated the phenylephrine-mediated elevation of intracellular $Ca^{2+}$ produced by $\alpha_{1b}$-adrenoceptor-$\alpha_{11}$ and this was also the case for the fusion proteins containing both $\text{Tyr}^{356}\text{Phe}\alpha_{11}$ and $\text{Tyr}^{356}\text{Ser}\alpha_{11}$ (data not shown).
As EF88 cells are difficult to transfect efficiently many of the subsequent studies were performed in HEK293 cells. The transfected $\alpha_{1b}$-adrenoceptor-$G\alpha_{11}$ fusion proteins were detected in lysates of HEK293 cells as distinct doublets that probably reflect differential glycosylation of the receptor element of the polypeptides (Figure 3A). Each of the $\alpha_{1b}$-adrenoceptor-$G\alpha_{11}$ fusion constructs containing the mutated forms of the G protein expressed as well as the fusion containing the wild type G protein as assessed by immunoblotting HEK293 cell lysates with an antiserum that identifies the extreme N-terminus of the $\alpha_{1b}$-adrenoceptor (Figure 3A, top). This was apparently not true when the same lysates were immunoblotted with an antiserum (CQ) that identifies the C-terminal decapetide of $G\alpha_{11}$. Only the construct containing the wild type G protein was identified effectively (Figure 3A, bottom). Unlike EF88 cells, HEK293 cells express both $G\alpha_q$ and $G\alpha_{11}$ [14]. Thus, although antiserum CQ was unable to identify the fusion proteins containing the G protein mutants it did identify the $G\alpha_q/G\alpha_{11}$ endogenously expressed in each sample (Figure 3A, bottom). Furthermore, ligand binding studies monitoring the specific binding of the $\alpha_1$-adrenoceptor antagonist $[^3H]$prazosin confirmed that each fusion protein was expressed to similar levels (Figure 3B). As the alteration in the G protein sequence was within the epitope used as antigen it is thus likely that Tyr$^{356}$ is a immunodominant element for identification of $G\alpha_{11}$ by antiserum CQ (see also later).

HEK293 membranes expressing 100 fmol of the $\alpha_{1b}$-adrenoceptor-wild type $G\alpha_{11}$ fusion protein, as measured by the specific binding of $[^3H]$prazosin, were subjected to a $[^35S]GTP\gamma S$ binding assay followed by immunoprecipitation with the anti-$G\alpha_q/G\alpha_{11}$ C-terminal antiserum. Very little $[^35S]GTP\gamma S$ was present in the immunoprecipitate (Figure 4). However, addition of phenylephrine during the assay increased the levels of $[^35S]GTP\gamma S$ present in the immunoprecipitate in a concentration-dependent manner. A maximally effective concentration of phenylephrine increased the level of bound $[^35S]GTP\gamma S$ nearly 30 fold with EC$_{50}$ for phenylephrine of $3.3 \times 10^{-7}$M (Figure 4A). Equivalent results were obtained when the experiments were repeated but immunoprecipitation performed with the antibody directed towards the N-terminus of the $\alpha_{1b}$-adrenoceptor. The EC$_{50}$ for phenylephrine was now $6.0 \times 10^{-7}$ M and although a lower number of counts of $[^35S]GTP\gamma S$ were present in these immunoprecipitates (Figure 4B) this simply reflects the immunoprecipitation efficiencies achieved with the amounts of the two antibodies employed (data not shown).
Further fusion proteins were constructed between the $\alpha_{1b}$-adrenoceptor and forms of $\mathrm{G}\alpha_{11}$ with each of Trp, Ile, Ala, Gln and Arg at position $^{356}$ of the G protein. These also were identified poorly by antiserum CQ but were expressed at similar levels as the other fusion proteins as monitored by saturation binding assays using [$^3$H]prazosin. Membrane amounts corresponding to 100fmol of the $\alpha_{1b}$-adrenoceptor-$\mathrm{G}\alpha_{11}$ fusion proteins containing Ser or Trp as residue $^{356}$ of the G protein were then used in [$^{35}$S]GTP$\gamma$S binding assays employing a range of concentrations of phenylephrine. Samples were immunoprecipitated with the anti-$\alpha_{1b}$-adrenoceptor antibody and counted (Figure 4B). Although the effectiveness of phenylephrine to stimulate nucleotide binding to the $\alpha_{1b}$-adrenoceptor-$\mathrm{G}\alpha_{11}$ fusion proteins containing Trp$^{356}$G$\alpha_{11}$ and particularly Ser$^{356}$G$\alpha_{11}$ was substantially lower than for the wild type (Figure 4B) this did not reflect a reduction in potency of the agonist. There was less than a 2 fold difference in the EC$_{50}$ for phenylephrine between the three constructs (Figure 4B). Each of the nine fusion proteins were then expressed in HEK293 cells and following saturation [$^3$H]prazosin binding assays to determine expression levels, membrane amounts containing 100fmol of each construct were used to measure basal and phenylephrine-stimulated [$^{35}$S]GTP$\gamma$S binding. Samples were subsequently immunoprecipitated with the anti-$\alpha_{1b}$-adrenoceptor antibody and counted (Figure 5A). A clear pattern emerged with the profile of [$^{35}$S]GTP$\gamma$S binding being that Tyr>Phe>Trp>Ile>Ala,Gln,Arg>Ser>Asp. Most effective guanine nucleotide exchange was obtained with amino acids containing an aromatic ring in the side chain. This did not reflect differences in the affinity of phenylephrine to bind to the individual constructs (Figure 5B, 5C). However, results were not the same when such [$^{35}$S]GTP$\gamma$S binding assays were subjected to immunoprecipitation with the anti-$\mathrm{G}\alpha_q$/G$\alpha_{11}$ antiserum CQ. Most of the constructs apparently functioned very poorly compared to the wild type sequence (data not shown). However, as noted earlier, this reflects that the various fusion proteins are not immunoprecipitated or immunodetected equally by this antiserum (see Figure 3B).

As Ca$^{2+}$ elevation in EF88 cells is dependent upon $\beta/\gamma$ release we generated a form of the $\alpha_{1b}$-adrenoceptor-$\mathrm{G}\alpha_{11}$ fusion protein by mutation of Gly$^{208}$ of the G protein to Ala. This was anticipated to be unable to exchange guanine nucleotide and thus should not be able to release $\beta/\gamma$ in an agonist-dependent manner. We also mutated residues in $\mathrm{G}\alpha_{11}$ (Ile$^{26}$Ala, Glu$^{26}$Ala) in a region shown previously [25] to be a
key β/γ contact region for Gα11. Following co-transfection of either the wild type α1b-adrenoceptor-Gα11 fusion protein or α1b-adrenoceptor-(Ile25Ala, Glu26Ala) Gα11 with a combination of the G protein β1 and γ2 subunits cell lysates were immunoprecipitated with the anti-Gαq/Gα11 antiserum. These samples were resolved by SDS-PAGE and immunoblotted to detect the β1 subunit (Figure 6 top). This was effectively co-immunoprecipitated with the wild type α1b-adrenoceptor-Gα11 fusion protein but it was hardly detectable following immunoprecipitation of the α1b-adrenoceptor-(Ile25Ala, Glu26Ala) Gα11 fusion protein (Figure 6 top). This was not a reflection of poor expression of the β1 subunit with the α1b-adrenoceptor-(Ile25Ala, Glu26Ala) Gα11 fusion protein as direct immunoblots of the cell lysates demonstrated equal levels of this subunit in the two samples (Figure 6 middle). The α1b-adrenoceptor-(Ile25Ala, Glu26Ala) Gα11 fusion protein was also expressed as effectively as the fusion protein containing the wild type G protein (Figure 6 bottom). Neither the α1b-adrenoceptor-(Ile25Ala, Glu26Ala) Gα11 nor the α1b-adrenoceptor-(Gly208Ala) Gα11 fusion protein caused effective elevation of intracellular [Ca2+] in response to phenylephrine following expression in EF88 cells (Figure 7).

We have previously demonstrated that the α1b-adrenoceptor-(Gly208Ala) Gα11 fusion protein is unable to bind [35S]GTPγS in response to phenylephrine [39]. Following expression in HEK293 cells and [3H]prazosin binding studies 100fmol of both wild type and the α1b-adrenoceptor-(Ile25Ala, Glu26Ala) Gα11 fusion construct were again used in [35S]GTPγS binding studies with subsequent immunoprecipitation. For these experiments the anti-G protein antiserum was used as the C-terminal region of the G protein is not different between these two constructs and, as noted earlier, this antiserum is the more efficient in immunoprecipitation if the C-terminal of the G protein is wild type. Although α1b-adrenoceptor-(Ile25Ala, Glu26Ala) Gα11 clearly bound [35S]GTPγS in response to a maximally effective concentration of phenylephrine (10μM) it was to a markedly lower level than the wild type fusion protein (Figure 8A). To explore the α1b-adrenoceptor-(Ile25Ala, Glu26Ala) Gα11 construct further, the time course of phenylephrine-stimulated [35S]GTPγS binding was compared to the α1b-adrenoceptor-Gα11 fusion. Phenylephrine-stimulated [35S]GTPγS binding to the α1b-adrenoceptor-(Ile25Ala, Glu26Ala) Gα11 fusion protein was substantially lower at all time points measured (Figure 8B). Co-expression of α1b-adrenoceptor-Gα11 with β1 and γ2 subunits did not significantly increase phenylephrine-mediated binding.
of \[^{35}S\]GTP\(\gamma\)S to the wild type fusion protein (Figures 8A, 8B). By contrast, co-expression of \(\beta_1\) and \(\gamma_2\) with the \(\alpha_{1b}\)-adrenoceptor-(Ile\(^25\)Ala, Glu\(^26\)Ala) G\(\alpha_{11}\) fusion protein did increase agonist-stimulated \[^{35}S\]GTP\(\gamma\)S binding to this fusion protein, although this remained lower than for the construct containing the wild type G protein (Figures 8A, 8B). It was not possible to assess whether co-expression of \(\beta_1\) and \(\gamma_2\) subunits with the \(\alpha_{1b}\)-adrenoceptor-(Ile\(^25\)Ala, Glu\(^26\)Ala) G\(\alpha_{11}\) fusion protein would allow agonist-mediated elevation of Ca\(^{2+}\) levels in EF88 cells. Expression of \(\beta_1/\gamma_2\) with or without the fusion protein resulted in spontaneous elevation of [Ca\(^{2+}\)]\(i\) (data not shown). This presumably reflects that significant amounts of the introduced \(\beta_1/\gamma_2\) did not become associated with G protein \(\alpha\) subunits and thus were active signal transducing complexes in the absence of receptor stimulation. Despite these differences \(\alpha_{1b}\)-adrenoceptor-(Ile\(^25\)Ala, Glu\(^26\)Ala) G\(\alpha_{11}\) bound both phenylephrine (Figure 8C) and \[^3\]H]prazosin (Figure 8D) with similar affinities as the construct containing the wild type G protein and these characteristics were not altered significantly by co-expression of \(\beta_1/\gamma_2\) (Figures 8C, 8D).

Discussion

The extreme C-terminus of G protein \(\alpha\) subunits provides a key contact interface for GPCRs and thus modification of this region is expected to alter either the effectiveness of information transfer or receptor selectivity [27-28]. This knowledge has been applied to the production of chimeric G proteins in which alteration of as few as the last 3 amino acids of G\(\alpha_q\) to the sequence of G\(\alpha_i\) has been shown to alter the classes of receptors able to activate the G protein [29-32]. Such chimeric G proteins are widely used in drug discovery programmes as they can allow a single, easy to measure, end-point assay to be used to infer activation of different classes of GPCRs [31-32]. Detailed studies of the effectiveness of interactions between GPCRs and G proteins of the pertussis toxin-sensitive G\(_i\) class have often taken advantage of the relatively high rate of both basal and agonist-stimulated guanine nucleotide exchange of these G proteins. By contrast, efforts to employ this approach for other G protein classes are generally limited by the poor signal to noise that is obtained. By immunoprecipitation of a fusion protein between the \(\alpha_{1b}\)-adrenoceptor and G\(\alpha_{11}\) at termination of a \[^{35}S\]GTP\(\gamma\)S binding assay we have shown that agonists produce a large
increase in binding of this nucleotide and that in this format the extremely low basal nucleotide exchange on Gα₁₁ is a distinct advantage [14].

Previous studies have suggested that the presence of the Tyr four amino acids from the C-terminus of Gα₉ and Gα₁₁ is integral to activation and indeed that this amino acid may well be a target for tyrosine phosphorylation [13]. Using either co-transfection of a receptor and mutants of this site in Gα₁₁ or by constructing a series of fusion proteins between the α₁β-adrenoceptor and variants of Gα₁₁ modified at this position we now show that this is not the case. Although Tyr is indeed the most effective amino acid at that location in allowing activation of the G protein, either Phe or Trp are well tolerated (Figure 5). As no other amino acid we tested was more than 40% as effective as Tyr there is, however, a clear requirement for a bulky and aromatic structure at this position. Amino acids with small side chains, such as Ser, or either positive or negative charge were poorly tolerated (Figure 5). In initial studies we were able to immunoprecipitate the wild type α₁β-adrenoceptor-Gα₁₁ fusion protein with antibodies directed against either the N-terminal region of the receptor or the C-terminal decapptide of the G protein. These produced equivalent data for the EC₅₀ of phenylephrine as agonist at the construct (Figure 4). However, when we repeated such experiments using the range of fusion proteins incorporating alterations in Tyr³⁵⁶ of the G protein it was clear that the anti-G protein antibody was not equally effective in identifying each fusion protein and thus could not be used to compare the effectiveness of agonist-induced [³⁵S]GTPγS binding. This antibody was generated against the G protein C-terminal peptide bound to a carrier protein via an N-terminal Cys. In such approaches bulky amino acids close to the C-terminal of the peptide sequence used as antigen are likely to be immunodominant and contribute significantly to the interaction between antibody and antigen. Indeed, we have noted previously that ADP-ribosylated Gαᵢ is a higher affinity target for an antibody against the C-terminal decapptide of this G protein that the unmodified sequence even though the native sequence was the antigen [33]. As the N-terminus of each fusion protein used herein is identical we used the anti-receptor antibody for the immunoprecipitation studies that were designed to determine the effect of Tyr³⁵⁶ mutation on signal transduction, even although the immunoprecipitation efficiency of this antiserum was lower than for the anti-G protein antiserum when this was assessed in parallel on the wild type fusion construct (Figure 4). The anti-Gα₉/Gα₁₁ antiserum used herein, and all those widely available from commercial sources, are directed against the C-terminal tail of
these polypeptides. It would not thus have been possible to quantitate the effect of mutants in this region of
G\(\alpha_{11}\) on information transfer from a receptor without constructing the receptor-G protein fusions used
herein because linking the G protein to the receptor was the best practical means to immunoprecipitate, and
hence enrich, the mutant forms of G\(\alpha_{11}\) at the termination of \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding assays. Although
immunoprecipitation of G\(\alpha_q/G\alpha_{11}\) with C-terminal anti-G protein antisera has been used previously to
enrich \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding assays [34-36] we show herein that this is only practical in receptor and G
protein co-transfection experiments that do not analyse such mutants of the G proteins. The major
limitation in the current studies was that many of the mutations we wished to study were within the epitope
identified by the antiserum. However, mutations also frequently alter the expression levels of a
polypeptide. The fusion protein approach also overcame this concern as we measured levels of expression
of each construct in ligand binding assays and thus were able to add the same amount of each distinct
construct to the \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding studies.

G proteins are activated by the exchange of GTP for GDP [1]. We thus also tested directly downstream
signal transduction via these fusion proteins. EF88 cells are derived from a G\(\alpha_q/G\alpha_{11}\) double knock-out
mouse and thus require expression of both an appropriate GPCR and a signal transduction competent G
protein to elevate intracellular Ca\(^{2+}\) in response to agonist [14, 18]. We have previously shown that a
fusion protein between the \(\alpha_{1b}\)-adrenoceptor and wild type G\(\alpha_{11}\) is functional [14]. The rank order of
capacity of fusions with alterations at Tyr\(^{356}\) of the G protein to elevate Ca\(^{2+}\) in response to phenylephrine
was the same as observed for the agonist-stimulated binding of \[^{35}\text{S}]\text{GTP}\gamma\text{S}\). We also noted that mutant
forms of G\(\alpha_{11}\) that coupled less effectively to the receptor as measured in the \[^{35}\text{S}]\text{GTP}\gamma\text{S}\) binding assays
displayed slower kinetics of elevation of [Ca\(^{2+}\)] in both co-transfection and fusion protein expression
studies (Figures 1 and 2). This is likely a reflection of the reduced effectiveness of information transfer and
thus activation of these mutants as they were expressed equally well as the wild type G protein (Figure 3).
A key point about agonist-mediated elevation of Ca\(^{2+}\) in EF88 cells is that it is blocked by co-expression of
\(\alpha\) transducin. As such, it is a measure of \(\beta/\gamma\) release and function [14]. It was thus anticipated that
incorporation of a \(\beta/\gamma\) release-deficient G protein into the fusion protein would result in a construct unable
to signal. Mutation of a conserved Gly in the G3 nucleotide binding domain of G protein \(\alpha\) subunits to Ala
is known to prevent $\beta/\gamma$ release from the $\alpha$ subunit, although this alteration has little obvious effect on G protein structure [37-38]. Mutations centred at Ile$^{25}$ and Glu$^{26}$ of Go$_{4}$ have previously been inferred to lack the capacity to bind $\beta/\gamma$ effectively [25]. Fusion proteins between the $\alpha_{1b}$-adrenoceptor and both wild type and Ile$^{25}$Ala,Glu$^{26}$Ala Go$_{11}$ were co-expressed with $\beta_{1}$ and $\gamma_{2}$ subunits and following immunoprecipitation of the fusion proteins, co-immunoprecipitation of the $\beta_{1}$ subunit was assessed. This polypeptide was shown to be associated with the wild type G protein containing fusion but only small amounts of $\beta_{1}$ were present along with the Ile$^{25}$Ala,Glu$^{26}$Ala Go$_{11}^{-}$containing fusion (Figure 6). Such results confirm the poor ability of Ile$^{25}$Ala,Glu$^{26}$Ala Go$_{11}$ to interact with the $\beta_{1}/\gamma_{2}$ complex. The Ile$^{25}$Ala,Glu$^{26}$Ala Go$_{11}$ containing fusion was only able to elevate Ca$^{2+}$ very poorly in EF88 cells in response to phenylephrine and even this was not observed in all transfected cells. Although this was also the case for the fusion containing Gly$^{208}$Ala Go$_{11}$ in this case it rather reflects that this construct was also unable to bind $[^{35}\text{S}]\text{GTP}_{\gamma}$S [39] and thus adopt the conformation required to dissociate the G protein subunits.

As $\beta/\gamma$ complex is also key for agonist-mediated guanine nucleotide exchange on the $\alpha$ subunit [16] we tested this quantitatively. Co-expression of the $\beta_{1}/\gamma_{2}$ complex with the fusion protein containing the wild type protein did not result in higher levels of phenylephrine-stimulated $[^{35}\text{S}]\text{GTP}_{\gamma}$S binding when the same number of fusion protein receptor binding sites were added to the assay. This is likely to reflect good interactions between the transfected fusion protein and endogenously expressed $\beta/\gamma$ complexes. Indeed, phenylephrine-mediated elevation of [Ca$^{2+}$] in EF88 cells (Figure 2) could not occur via the wild type fusion protein without this interaction. However, although the fusion protein containing Ile$^{25}$Ala,Glu$^{26}$Ala Go$_{11}$ bound significantly less $[^{35}\text{S}]\text{GTP}_{\gamma}$S than the wild type, phenylephrine-stimulated binding of the nucleotide was increased to this fusion protein in the presence of co-expressed $\beta_{1}/\gamma_{2}$. This is likely to reflect that Ile$^{25}$Ala,Glu$^{26}$Ala Go$_{11}$ has a significantly lower affinity to bind $\beta_{1}/\gamma_{2}$ than wild type Go$_{11}$ (and thus was poor in producing co-immunoprecipitation) rather than being entirely lacking in this regard. These studies expand the recent use [14] of a receptor-G protein fusion protein to overcome the traditional difficulties in monitoring directly guanine nucleotide exchange on G$_{q}$ family G proteins to allow analysis of the importance of both $\beta/\gamma$ interactions and the role of Tyr$^{356}$ in effective information transfer between a
receptor and Go_{11}. This basic approach should be equally amenable to any sets of mutations in receptors and G proteins from these families.

Acknowledgement

We thank the Medical Research Council and the Wellcome Trust for financial support. The anti-\alpha_1b-adrenoceptor antiserum was a gift from Susanna Cotecchia, University of Lausanne, Switzerland.

References

1. Gilman, A.G. (1987) *Ann. Rev. Biochem.* **56**, 615-647.
2. Wieland, T. and Jakobs, K.H. (1994) *Meth. Enzymol.* **237**, 1-13.
3. Windh, R.T., Lee, M.J., Hla, T., An, S., Barr, A.J. and Manning, D.R. (1999) *J. Biol. Chem.* **274**, 27351-27358.
4. Strathmann, M., and Simon, M.I. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9113-9117.
5. Wilkie, T.M., Scherle, P.A., Strathmann, M.P., Slepak, V.Z., and Simon, M.I. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10049-10053.
6. Simon, M.I., Strathmann, M.P. and Gautam, N. (1991) *Science* **252**, 802-808.
7. Taylor, S.J., Chae, H.Z., Rhee, S.G. and Exton, J.H. (1991) *Nature* **350**, 516-518.
8. Blank, J.L., Ross, A.H. and Exton, J.H. (1991) *J. Biol Chem.* **266**, 18206-18216.
9. Gutowski, S., Smrcka, A., Nowak, L., Wu, D.G., Simon, M. and Sternweis, P.C. (1991) *J. Biol. Chem.* **266**, 20519-20522.
10. Milligan, G. (1998) *Biochem. J.* **255**, 1-13.
11. Bahia, D.S., Wise, A., Fanelli, F., Lee, M., Rees, S. and Milligan, G. (1998) *Biochemistry* **37**, 11555-11562.
12. Dupuis, D.S., Wurch, T., Tardif, S., Colpaert, F.C. and, Pauwels, P.J. (2001) *Neuropharmacology* **40**, 36-47.
13. Umemori, H., Inoue, T., Kume, S., Sekiyama, N., Nagao, M., Itoh, H., Nakanishi, S., Mikoshiba, K. and Yamamoto, T. (1997) *Science* **276**, 1878-1881.
14. Stevens, P.A., Pediani, J., Carrillo, J.J. and Milligan, G. (2001) *J. Biol. Chem.* **276**, 35883-35890.
15. Azpiazu, I. and Gautam, N. (2001) *J. Biol. Chem.* **276**, 41742-41747.

16. Rondard, P., Iiri, T., Srinivasan, S., Meng, E., Fujita, T. and Bourne, H.R. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 6150-6155.

17. Mao, J., Yuan, H., Xie, W., Simon, M.I. and Wu, D. (1998) *J. Biol. Chem.* **273**, 27118-27123.

18. Yu, R. and Hinkle, P.M. (1999) *J. Biol. Chem.* **274**, 15745-15750.

19. Gohla, A., Offermanns, S., Wilkie, T.M. and Schultz, G. (1999) *J. Biol. Chem.* **274**, 17901-17907.

20. Offermanns, S., Zhao, L.P., Gohla, A., Sarosi, I., Simon, M.I. and Wilkie, T.M. (1998) *EMBO J.* **17**, 4304-4312.

21. Offermanns, S. (2001) *Oncogene* **20**, 1635-1642.

22. Mitchell, F.M., Buckley, N.J. and Milligan, G. (1993) *Biochem. J.* **293**, 495-499.

23. Kim, G.-D., Carr, I.C., Anderson, L.A., Zabavnik, J., Eidne, K.A. and Milligan, G. (1994) *J. Biol. Chem.* **269**, 19933-19940.

24. Stevens, P.A., Bevan, N., Rees, S., and Milligan, G. (2000) *Mol. Pharmacol.* **58**, 438-448.

25. Evanko, D.S., Thiyagarajan, M.M. and Wedegaertner, P.B. (2000) *J. Biol. Chem.* **275**, 1327-1336.

26. Tsiens, R.Y. and Harootunian, A. (1990), *Cell Calcium.* **11**, 93-109.

27. Hamm, H.E. (1998) *J. Biol. Chem.* **273**, 669-672.

28. Wess, J. (1998) *Pharmacol. Ther.* **80**, 231-264.

29. Conklin, B.R., Farfel, Z., Lustig, K.D., Julius, D. and Bourne, H.R. (1993) *Nature* **363**, 274-276.

30. Conklin, B.R., Herzmark, P., Ishida, S., Vino-Vykonetska, T.A., Sun, Y., Farfel, Z. and Bourne, H.R. (1998) *Mol. Pharmacol.* **50**, 885-890.

31. Milligan, G. and Rees, S. (1999) *Trends Pharmacol. Sci.* **20**, 118-124.

32. Kostenis, E. (2001) *Trends Pharmacol. Sci.* **22**, 560-564.

33. Goldsmith, P., Gierschik, P., Milligan, G., Unson, C.G., Vinitsky, R., Malech, H. and Spiegel, A. (1987) *J. Biol. Chem.* **262**, 14683-14688.

34. DeLapp, N.W., McKeinzie, J.H., Sawyer, B.D., Vandergriff, A., Falcone, J., McClure, D. and Felder, C.C. (1999) *J. Pharmacol. Exp. Ther.* **289**, 946-955.

35. Akam, E.C., Challiss, R.A. and Nahorski, S.R. (2001) *Br. J. Pharmacol.* **132**, 950-958.

36. Willets, J.M., Challiss, R.A., Kelly, E. and Nahorski, S.R. (2001) *Mol. Pharmacol.* **60**, 321-330.
37. Sprang, S.R. (1997) *Ann. Rev. Biochem.* **66**, 639-678.

38 Wall, M.A., Coleman, D.E., Lee, E., Iniguez-Lluhi, J.A., Posner, B.A., Gilman, A.G. and Sprang, S.R. (1995) *Cell* **83**, 1047-1058.

39. Carrillo, J.J., Stevens, P.A. and Milligan, G. (2002) *J. Pharmacol Exp. Ther.* (in press).

40. Lattion, A.L., Diviani, D. and, Cotecchia, S. (1994) *J. Biol. Chem.* **269**, 22887-22893.
**Figures**

**Figure Legends**

**Figure 1. Mutation of Tyr$^{356}$ of $\alpha_{11}$ alters interaction with the $\alpha_{1b}$-adrenoceptor.**

EF88 cells were transfected to express the $\alpha_{1b}$-adrenoceptor along with either wild type (Tyr$^{356}$) $\alpha_{11}$ (black) or mutants of $\alpha_{11}$ in which Tyr$^{356}$ was replaced by Phe (red), Ser (green) or Asp (blue). In some experiments with the $\alpha_{1b}$-adrenoceptor and wild type $\alpha_{11}$ cells were transfected to also express transducin $\alpha$ (pink). Green fluorescent protein was co-expressed as a marker of positively transfected cells. Cells were loaded with Fura2/AM and $[Ca^{2+}]_{i}$ levels imaged before and during exposure to phenylephrine (Phe, 3$\mu$M). Data represent means + S.E.M. from analysis of 24 (wild type), 18 (Phe$^{356}$), 18 (Ser$^{356}$), 20 (Asp$^{356}$) and 10 (wild type plus transducin $\alpha$) positively transfected cells.

**Figure 2. Fusion proteins between the $\alpha_{1b}$-adrenoceptor and forms of $\alpha_{11}$: effects of alteration of Tyr$^{356}$$\alpha_{11}$ on signal transduction.**

EF88 cells were transfected to express fusion proteins between the $\alpha_{1b}$-adrenoceptor and each of wild type (black), Phe$^{356}$ (red), Ser$^{356}$ (green) or Asp$^{356}$ (blue) $\alpha_{11}$. Positively transfected cells were identified by co-expression of green fluorescent protein. Cells were loaded with Fura2/AM and $[Ca^{2+}]_{i}$ levels imaged over time following exposure to 3$\mu$M phenylephrine (Phe). Data represent means + S.E.M. from analysis of 27 (wild type), 16 (Phe$^{356}$), 17 (Ser$^{356}$) and 20 (Asp$^{356}$) positively transfected cells. Representative IMD ratio images are shown of cells expressing the $\alpha_{1b}$-adrenoceptor-$\alpha_{11}$ (WT) and $\alpha_{1b}$-adrenoceptor-Ser$^{356}$$\alpha_{11}$ (S) fusion proteins before addition of agonist and at maximal $[Ca^{2+}]_{i}$. Higher $[Ca^{2+}]_{i}$ are represented by warmer colours.

**Figure 3. Mutations of $\alpha_{11}$ at Tyr$^{356}$ limit detection by a C-terminal anti $\alpha_{11}/\alpha_{q}$ antiserum.**

A. HEK293 cells were transfected to express fusion proteins between the $\alpha_{1b}$-adrenoceptor and each of wild type ($Y$) or Phe$^{356}$ ($F$), Ser$^{356}$ ($S$) or Asp$^{356}$ ($D$) $\alpha_{11}$. Lysates of these cells were resolved by SDS-
PAGE and then immunoblotted (WB) with an antiserum directed towards the N-terminal segment of the α₁b-adrenoceptor [ref. 40] (top) or with the C-terminal anti Gα₁/Gα₁₅ antiserum, CQ [ref. 23] (bottom).

**B.** Membranes produced from these cells were used to measure the specific binding of [³H]prazosin (1 nM) to fusion proteins containing wild type (Y), Phe³⁵⁶ (F), Ser³⁵⁶ (S) or Asp³⁵⁶ (D) Gα₁₁.

**Figure 4.** Phenylephrine stimulates binding of [³⁵S]GTPγS to α₁b-adrenoceptor-Gα₁₁ fusion proteins.

Membranes expressing 100 fmol of the α₁b-adrenoceptor-wild type Gα₁₁ fusion protein (circles) or equivalent fusions in which residue ³⁵⁶ of the G protein was Trp (squares) or Ser (diamonds) were added to [³⁵S]GTPγS binding assays in the presence of a range of concentrations of phenylephrine. Samples were subsequently immunoprecipitated with antiserum CQ (A) or with the antiserum directed to the N-terminus of the α₁b-adrenoceptor (B) and counted.

**Figure 5.** The identity of residue³⁵⁶ of Gα₁₁ determines the effectiveness of phenylephrine to stimulate binding of [³⁵S]GTPγS to α₁b-adrenoceptor-Gα₁₁ fusion proteins.

α₁b-adrenoceptor-Gα₁₁ fusion proteins in which residue³⁵⁶ of the G protein was Tyr, Phe, Trp, Ile, Ala, Gln, Arg, Ser or Asp were expressed in HEK293 cells. A. Membranes were prepared and the specific binding of [³H]prazosin measured. 100fmol of each construct were used in [³⁵S]GTPγS binding assays in the absence (open bars) or presence (filled bars) of 100μM phenylephrine. Samples were subsequently immunoprecipitated with the antiserum to the N-terminus of the α₁b-adrenoceptor and counted. Amino acids at position³⁵⁶ of Gα₁₁ are represented by the standard one letter code. B, C. The capacity of varying concentrations of phenylephrine to compete with [³H]prazosin (1.0 nM) for binding to the fusion constructs was assessed. Tyr (open circles), Phe (open squares), Trp (filled squares), Ile (open inverted triangles), Ala (open diamonds), Gln (filled diamonds), Arg (filled triangles), Ser (inverted filled triangles) or Asp (open triangles).

**Figure 6.** Interaction of β₁/γ₂ complex with the α₁b-adrenoceptor-Gα₁₁ fusion protein is greatly reduced by mutation of Ile²⁵ and Gln²⁶ of Gα₁₁.
HEK293 cells were mock transfected (1) or transfected to express both the β1 and γ2 G protein subunits (2-3) along with either the wild type α1b-adrenoceptor-Gα11 fusion protein (2) or a variant of this in which Ile25 and Gln26 of Gα11 were converted to Ala (3). Lysates of these cells were immunoprecipitated (IP) with antiserum CQ and samples resolved by SDS-PAGE and then immunoblotted to detect the presence of the β1 subunit (top). Lysates of the cells were also resolved directly by SDS-PAGE and immunoblotted to detect the expression of the β1 subunit (middle) or the fusion proteins (bottom).

Figure 7. Fusion proteins that fail to bind or release β1/γ2 complex do not elevate [Ca2+]i effectively in EF88 cells.

EF88 cells were transfected to express the wild type α1b-adrenoceptor-Gα11 fusion protein (black) or variants of this (α1b-adrenoceptor-Ile25Ala, Gln26Ala Gα11 ) (green) that fail to bind β1/γ2 efficiently or (α1b-adrenoceptor-Gly208Ala Gα11 ) that fails to release it (red). The effects of phenylephrine. (3µM) on [Ca2+]i were then imaged as in Figure 2. Data represent means + S.E.M. from analysis of 27 (wild type G11α), 10 (Gly208Ala Gα11) and 19 (Ile25Ala, Gln26AlaGα11) positively transfected cells.

Figure 8. Contacts with β/γ are required for effective information transfer from the α1b-adrenoceptor to Gα11.

HEK293 cells were transfected to express the wild type α1b-adrenoceptor-Gα11 fusion protein (WT and circles) or the α1b-adrenoceptor-Ile25Ala, Gln26Ala Gα11 fusion protein (IE and squares) in the absence (open symbols) or presence (βγ and closed symbols) of β1 and γ2 subunits. ** Significantly different from wild type. * Significantly different from from IE and from wild type plus β/γ.

A. 100fmol of each construct was used in [35S]GTPγS binding assays in the absence (open bars) or presence (filled bars) of phenylephrine (100µM). Samples were subsequently immunoprecipitated with antiserum CQ and counted.

B. 100fmol of each construct were used in [35S]GTPγS binding assays in the absence or presence of phenylephrine (100µM). Samples were subsequently immunoprecipitated with antiserum CQ after various times of incubation and counted.
C. Membranes expressing various combinations of fusion proteins and β₁ and γ₂ were prepared and the ability of varying concentrations of phenylephrine to compete with [³H]prazosin (1.0 nM) for binding assessed.

D. Membranes as in C were prepared and the specific binding of varying concentrations of [³H]prazosin measured.
Figure 1

![Graph showing Fura-2 fluorescence ratio](image-url)

- Fura-2 fluorescence ratio
- + Phe (3 µM)
- 0.90
- 1.40
- 1.90
- 2.40
- 20 sec
Figure 2

Fura-2 fluorescence ratio

+ Phe (3 µM)

20 sec

WT

- Phe

+ Phe

S

- Phe

+ Phe
Figure 3

A

WB
α_{1b}AR

WB
Gα_q/Gα_{11}

Mr
(x10^3)

160
105

250
160
105
75
50
35

Y F D S

B

[^3]H prazosin binding
(fmol/mg)

0
2500
5000
7500
10000

Y F D S
Figure 4

A

GTP binding (cpm, dpm) vs. -Log[Phenylephrine] M

B

GTP binding (cpm, dpm) vs. -Log [Phenylephrine] (M)
Figure 5

A

B

C

GTP binding (3^5S dpm)

[^3]H prazosin binding (% of maximal)

[^3]H prazosin binding (% of maximal)
Figure 6

**IP** (CQ Ab)

Cell lysates

1 2 3

α/β

β

α/β

β
Figure 7

Fura-2 fluorescence ratio

+ Phe (3 µM)
Figure 8

A  
GTP binding (fold over basal WT)  
WT  | IE  | WT +βγ  | IE +βγ  
---|---|---|---  
25 | 10 | 5  | *  
20 | 15 | 10  | **  
15 | 5 | 5 |  
5 | 0  |  

B  
GTP binding (3H dpm)  
Time (min)  
0 | 5 | 10 | 15 | 20 | 25 | 30  
---|---|---|---|---|---|---  
8000 | 6000 | 4000 | 2000 |  
6000 | **  |  
4000 |  
2000 |  

C  
[3H] prazosin binding (% of maximal)  
-Log [Phenylephrine] M  
10 | 9 | 8 | 7 | 6 | 5 | 4 | 3  
---|---|---|---|---|---|---|---  
125 | 110 | 100 | 90 | 80 | 70 | 60 | 50 | 40 | 30 | 20 | 10 | 0  

D  
[3H] Prazosin binding (fmol/mg)  
[Prazosin] nM  
0.0 | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0  
---|---|---|---|---|---|---  
8000 | 6000 | 4000 | 2000 |  
6000 |  
4000 |  
2000 |  

Effective information transfer from the α1b-adrenoceptor to Gα11 requires both β/γ interactions and an aromatic group four amino acids from the C-terminus of the G protein

Sen Liu, Juan J. Carrillo, John D. Pediani and Graeme Milligan

J. Biol. Chem. published online May 6, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M201015200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts