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Functional interactions between the α_{1b}-adrenoceptor and $G\alpha_{11}$ are compromised by de-palmitoylation of the G protein but not of the receptor

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Abstract

Both the α_{1b}-adrenoceptor and $G\alpha_{11}$ are targets for post-translational thio-acylation that is regulated by agonist occupancy of the receptor [P.A. Stevens, J. Pediani, J.J. Carrillo, G. Milligan, J. Biol. Chem. 276 (2001) 35883]. In co-expression studies mutation of the sites of thio-acylation in the G protein or treatment of cell membranes with hydroxylamine greatly reduced agonist stimulation of guanosine 5′-[γ-[^{35}S]thio]triphosphate ([^{35}S]GTPγS) binding. In α_{1b}-adrenoceptor-$G\alpha_{11}$ fusion proteins mutation of thio-acylation sites in receptor or G protein did not alter the binding affinity of the antagonist [^{3}H]prazosin or the agonist phenylephrine. Although the potency of phenylephrine to stimulate binding of [^{35}S]GTPγS to α_{1b}-adrenoceptor-$G\alpha_{11}$ fusion proteins was unaffected by the thio-acylation potential of either element, the maximal effect was reduced by some 50% when the G protein but not the receptor was mutated to prevent thio-acylation. This reflected lack of thio-acylation of the G protein rather than mutation of Cys^{9} and Cys^{10} to Ser because treatment with hydroxylamine mimicked this in fusions containing the wild type G protein but was without effect in those mutated to prevent thio-acylation. Mutation to reduce binding of β/γ to $G\alpha_{11}$ markedly reduced phenylephrine stimulation of [^{35}S]GTPγS binding. Combination of mutations to prevent thio-acylation and β/γ binding did not, however, have an additive effect on [^{35}S]GTPγS binding. These results indicate that the thio-acylation status of the α_{1b}-adrenoceptor does not regulate G protein activation whereas thio-acylation of $G\alpha_{11}$ plays a key role in activation by the receptor beyond providing membrane association and proximity.

Keywords: Palmitoylation; Heterotrimeric G protein; β/γ complex; Adrenoceptor

1. Introduction

Post-translational thio-acylation is an important contributor to the tertiary structure and cellular localization of both G protein-coupled receptors (GPCRs) and G proteins [1–3]. For the class A, rhodopsin-like GPCRs one or more cysteine residue in the C-terminal tail generally acts as the sites of thio-acylation [1]. In the crystal structure of rhodopsin the sequence between the end of transmembrane segment VII and the pair of thio-acylated cysteines forms an '8th helix' [4] and the cysteine-linked fatty acyl chains have been shown directly to insert into the membrane [5]. It is likely that these features will be conserved in other related GPCRs. This region has also been implicated in interactions with both the N- and C-terminal regions of G protein α subunits [6]. The lability of thio-ester acyl links is well established and in many cases agonist-occupation of a GPCR results in alteration in the thio-acylation status of the protein [1,7]. Mutation of the relevant cysteine residue(s) has been widely employed to explore the effects of preventing acylation of these sites. This has produced a complex literature in which both effects [8–13] and lack of effect [14] on G protein activation have been described. A number of such GPCR mutants have also been reported to display altered capacity to interact with β-arrestins and to undergo endocytosis [15–18].

Abbreviations: GPCR, G protein-coupled receptor; [^{35}S]GTPγS, guanosine 5′-[γ-[^{35}S]thio]triphosphate.

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Membrane association of the α subunits of heterotrimeric G proteins is provided by a complex combination of their interactions with other polypeptides, particularly the G protein β/γ complex, and both co- and post-translational acylation [2,3,19–21]. $G\alpha_{11}$ is a member of the G_q family of Ca^{2+}-mobilizing G proteins that is expressed in virtually all cells and tissues except for those of haemopoietic origin [22–24]. Previous studies have shown that both Cys^9 and Cys^{10} of $G\alpha_{11}$ are sites for post-translational acylation [25] and that alteration of both these residues to Ser eliminates such acylation and may prevent activation of the G protein by co-expressed GPCRs [26].

Fusion proteins in which the N-terminus of a G protein α subunit is attached in frame to the C-terminal tail of a GPCR have become popular reagents to study many aspects of the interactions between these classes of polypeptides [27,28]. The physical link imbued by the fusion ensures the proximity of GPCR and G protein and defines a 1:1 stoichiometry between them. This strategy allows analysis of the effects of mutation in either GPCR or G protein on information transfer between them without potentially confounding effects of alterations in proximity or relative expression levels. Following expression in HEK293 cells of a fusion protein between the hamster α_{1b}-adrenoceptor and $G\alpha_{11}$ this polypeptide becomes thio-acylated [29]. Mutational analysis showed this to occur in both the GPCR and G protein elements and at the residues anticipated from earlier work [29]. Furthermore, thio-acylation of both elements of the fusion protein was regulated upon agonist occupation of the fusion protein [29]. The current studies were undertaken to examine the contribution of thio-acylation of either GPCR or G protein to agonist-mediated G protein activation. We demonstrate that the effectiveness of G protein activation is enhanced by thio-acylation of the G protein but unaffected by the thio-acylation status of the receptor. Furthermore, although thio-acylation of G protein α subunits and interactions with β/γ complex are inter-related [19–21] combinations of mutations of $G\alpha_{11}$ to limit interactions with β/γ and prevent thio-acylation do not produce additive effects in limiting receptor-mediated activation of the G protein.

2. Materials and methods

2.1. Materials

All materials for tissue culture were supplied by Sigma (Gillingham, Dorset, U.K.). [^{35}S]GTPγS (1250 Ci/mmol) and [^{3}H]prazosin (80 Ci/mmol) were from NEN/Perkin Elmer. Oligonucleotides were purchased from ThermoHybaid (Ulm, Germany). The anti-$G\alpha_{11}$/ $G\alpha_q$ antiserum, CQ, has been described previously [30].

2.2. Construct generation

Generation and characterization of the wild type α_{1b}-adrenoceptor-$G\alpha_{11}$ fusion protein [29] and both $Cys^9Ser,Cys^{10}SerG\alpha_{11}$ [25] and $Ile^{25}Ala,Glu^{26}AlaG\alpha_{11}$ [31] have been described previously. The $Cys^9Ser,Cys^{10}Ser,Ile^{25}Ala,Glu^{26}$-$AlaG\alpha_{11}$ mutant was constructed by introducing via site directed mutagenesis the $Cys^9Ser,Cys^{10}Ser$ mutations into $Ile^{25}Ala,Glu^{26}AlaG\alpha_{11}$ which was subcloned into pcDNA3.0 (Invitrogen). This plasmid was used as a template for PCR using the primer 5′-CTG GAG TCC ATG ATG GCG TCT TCC CTG AGC GAC GAG GTG AAG-3′ and the complementary primer 5′-CTT CAC CTC GTC GCT CAG GGA AGA CGC CAT CAT GGA CTC CAG-3′. Following temperature cycling the product was treated with *Dpn*I to digest the methylated parental DNA. Finally the vector containing the mutations was transformed into XL1-Blue competent cells. The same procedure was carried out to construct the α_{1b}-adrenoceptor-$Cys^9Ser,Cys^{10}Ser,Ile^{25}Ala,Glu^{26}AlaG\alpha_{11}$ fusion protein, but using the α_{1b}-adrenoceptor-$Cys^9Ser,Cys^{10}SerG\alpha_{11}$ construct as template.

An internal HA epitope tag was introduced into forms of $G\alpha_{11}$ by making the following mutations $Glu^{125}Asp$, $His^{126}Val$, $Gln^{127}Pro$, $Tyr^{128}Asp$, $Val^{129}Tyr$ and $Asn^{130}Ala$. This is akin to an internally HA tagged form of $G\alpha_q$ described previously [19,20]. These mutations were made by an overlapping fragment PCR approach, where two PCR fragments were generated containing the mutations at the overlapping 5′ and 3′ ends, respectively. These fragments were then used as primers/templates with appropriate additional primers at the non-overlapping ends to amplify full-length mutant fragments. These fragments were then subcloned into $G\alpha_{11}$ by replacing the $G\alpha_{11}$ C-terminal as a *Sac*I–*Xho*I fragment.

2.3. Transient transfection of HEK293 cells

HEK293 cells were maintained in DMEM supplemented with 0.292g/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 60 mm dishes. Transfection was performed using LipofectAMINE reagent (Life Technologies, Inc.) according to the manufacturer's instructions.

2.4. [^{3}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

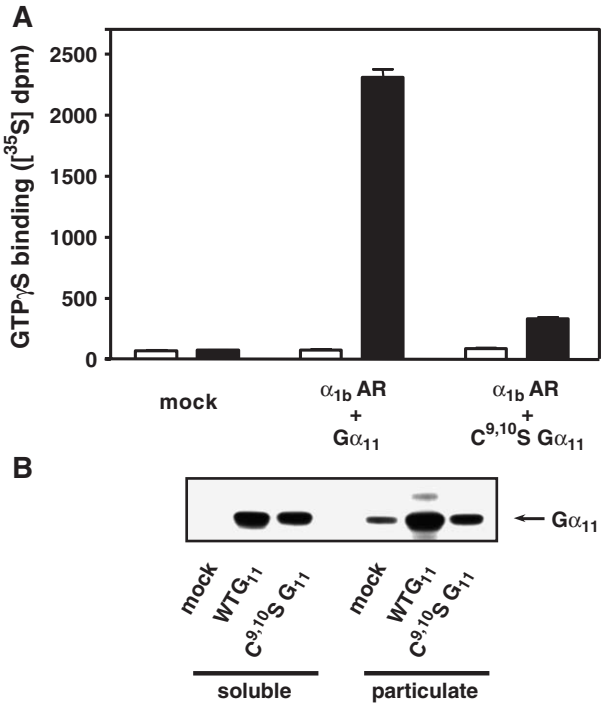

Fig. 1. α_{1b}-adrenoceptor stimulation of [^{35}S]GTPγS binding to $G\alpha_{11}$ is greatly reduced by mutations that prevent thio-acylation of the G protein. (A) HEK293 cells were mock transfected or transfected to express the α_{1b}-adrenoceptor along with either wild type $G\alpha_{11}$ or $Cys^9Ser,Cys^{10}SerG\alpha_{11}$ ($C^{9,10}SG\alpha_{11}$). Membranes from these cells were subjected to [^{35}S]GTPγS binding assays in the absence (open bars) or presence (filled bars) of 100 μM phenylephrine. Samples were immunoprecipitated with antiserum CQ and counted. Data are means±S.E.M. of triplicates of a single experiment repeated on three occasions. B. Cell lysates corresponding to the samples in panel A were resolved into particulate and soluble fractions by high speed centrifugation. Equivalent amounts were resolved by SDS-PAGE and immunoblotted using antiserum CQ. Endogenously expressed $G\alpha_{11}$ and/or $G\alpha_q$ are also identified by this antiserum.